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Alterations in pp60$^{c\text{-}src}$ Accompany Differentiation of Neurons from Rat Embryo Striatum

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Cultured neurons from rat embryo striatum were found to contain two structurally distinct forms of pp60$^{c\text{-}src}$. The 60-kilodalton (kDa) form appeared similar to pp60$^{c\text{-}src}$ from cultured rat fibroblasts or astrocytes. The 61-kDa form was specific to neurons and differed in the NH_2-terminal 18 kDa of the molecule. In undifferentiated neurons the predominant phosphorylated species of pp60$^{c\text{-}src}$ was the fibroblast form. Upon differentiation, a second phosphorylated form of pp60$^{c\text{-}src}$ was detected. This form had two or more additional sites of serine phosphorylation within the NH_2-terminal 18-kDa region of the molecule, one of which was Ser-12. The specific protein-tyrosine kinase activity of the total pp60$^{c\text{-}src}$ population increased 14-fold, as measured by autophosphorylation, or 7-fold, as measured by phosphorylation of an exogenous substrate, as striatal neurons differentiated. This elevation in protein kinase activity occurred without a detectable decrease in Tyr-527 phosphorylation or increase in Tyr-416 phosphorylation. Our results support the idea that the expression of the neuron-specific form of pp60$^{c\text{-}src}$ and the increase in specific protein kinase activity may be important for neuronal differentiation.

Cellular genes homologous to retroviral oncogenes are present in the genomes of all vertebrates. These cellular proto-oncogenes have been highly conserved throughout evolution, suggesting that they are essential to the organism. Although their function remains largely unknown, evidence is accumulating that some may be important for normal cell differentiation or growth.

The cellular gene c-*src* is homologous to the transforming gene of Rous sarcoma virus (59). It is highly conserved phylogenetically, being present in the genome of such widely divergent species as humans, *Drosophila melanogaster* (35, 61, 62), and the freshwater sponge *Spongilla lacustris* (3, 55). The c-*src* gene encodes a 60-kilodalton (kDa) membrane-associated phosphoprotein, pp60$^{c\text{-}src}$, which is a protein-tyrosine kinase (14–17, 24, 36, 38, 49, 52, 59). Evidence is emerging that pp60$^{c\text{-}src}$ is the product of a developmentally regulated gene that may participate in cell differentiation. High levels of pp60$^{c\text{-}src}$ are expressed in brain and other neural tissues of both chickens and humans during embryogenesis (22, 42, 45). Expression of pp60$^{c\text{-}src}$ in the developing chick neural retina (64) and cerebellum (29) coincides with the onset of neuronal differentiation. Post mitotic neurons from the central nervous system of rat embryos express high levels of a structurally distinct, enzymatically activated form of pp60$^{c\text{-}src}$ (9). Similarly, embryonal carcinoma cells induced to differentiate into neuronlike cells have elevated levels of a slower-migrating form of pp60$^{c\text{-}src}$ (44). There are other nonproliferating cells, for example, platelets (31) and myeloid cells (2, 30), which contain increased pp60$^{c\text{-}src}$ kinase activity. The presence of high levels of pp60$^{c\text{-}src}$ protein-tyrosine kinase activity in these nondividing cells suggests that pp60$^{c\text{-}src}$ may be important to induction or maintenance of the differentiated state.

Neurons provide a useful system for studying the possible role of pp60$^{c\text{-}src}$ in cell differentiation. Relatively pure populations of neurons can be maintained in culture and induced to differentiate under a variety of conditions. We chose to focus on neurons from a region of the brain with well-defined neuronanatomy, pathophysiology, and ontogeny. The striatum, or caudate putamen, has been extensively studied over the past 70 years (48, 63, 66, 69). It has a unique architectural organization (33, 70) and is a region rich in neurotransmitters and neuropeptides (28). The striatum has received considerable attention since the demonstration of its dopaminergic input from the substantia nigra and the effectiveness of the drug L-dopa in the treatment of parkinsonism. In rats, the striatum develops within a relatively short time span, primarily during gestation. The peak period of neuronogenesis precedes that of gliogenesis and occurs on embryonic day 16 (E16) or E17 (27, 70). We chose to study striatum from day 15 embryos to include cells predominantly of the neuronal lineage, which had not yet acquired the morphological, cytochemical, and physiological features of fully differentiated postmitotic neurons. We cultured striatal neurons in serum-free medium to induce differentiation (58, 68), which was defined morphologically by process extension.

Our results confirm previously published data showing a new form of pp60$^{c\text{-}src}$ in differentiated neurons, which is structurally distinct in its NH_2-terminal 18 kDa and has elevated protein kinase activity (6, 9, 44). In addition, we found that this form of pp60$^{c\text{-}src}$ in differentiated neurons contained at least two additional sites of serine phosphorylation in its NH_2-terminal region. Our results, like those obtained using an embryonal carcinoma cell line induced to differentiate into neuronlike cells with retinoic acid (44), show that there was an increase in pp60$^{c\text{-}src}$ protein kinase activity as neurons differentiated.

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MATERIALS AND METHODS

Cell culture. Striata were dissected under a binocular microscope from Sprague-Dawley day 15 embryos, and cells were dissociated by incubation for 7 to 8 min in 0.25% trypsin at 37°C. The enzyme solution was removed, and the tissue was washed three times with Ca^{2+}-Mg^{2+}-free Hanks balanced salt solution containing 57 μM phenylmethylsulfonyl fluoride. The tissue was then suspended in Dulbecco-Vogt modified Eagle medium (DME) and dissociated by trituration with a silane-coated glass pipette, and the pieces were removed by filtration through a nylon net. The cells were then counted, and 1×10^6 to 2×10^6 or 3×10^6 to 4×10^6 cells were plated in 60- or 100-mm plastic tissue culture dishes, respectively. The dishes were previously coated with polyornithine and washed as described (46). The culture medium was high-glucose (4.5 mg/ml) DME supplemented with 5 μg of insulin per ml, 100 μg of transferrin per ml, 2 mM glutamine, 100 μM putrescine, 30 nM selenium, 20 nM progesterone, 100 U each of penicillin and streptomycin per ml and 0.25 μg of amphotericin B per ml. (All chemicals and hormones added to the culture medium were purchased from Sigma Chemical Co.) During the first 4 or 5 days of culturing, the medium also contained 7.5% fetal calf serum (GIBCO Laboratories). Cultures were kept at 37°C in a humidified CO_2 incubator for the indicated period (2 to 18 days). Cultures of astrocytes were prepared from whole brains of 2-day-old rats. After removal of the meninges the brains were dissected into 3- to 5-mm pieces, dissociated enzymatically with trypsin, washed with Ca^{2+}-Mg^{2+}-free Hanks balanced salt solution containing phenylmethylsulfonyl fluoride and DNase (30 μg/ml), and plated at 5×10^5 cells per 60-mm dish. The culture medium, DME containing 7.5% fetal calf serum, was changed every 4 or 5 days, and the cells were used 15 days after seeding. Rat 208F cells were maintained at 37°C in DME containing 10% calf serum (GIBCO). The cells (2×10^6) were plated in 100-mm tissue culture dishes 18 h before labeling.

Immunohistochemistry. Neurons and glial cells were identified by their ability to bind tetanus toxin or to express the glial fibrillary acidic protein, respectively. Cultures were washed three times with phosphate-buffered saline, fixed with 4% paraformaldehyde, rewashed, and incubated with tetanus toxin (kindly provided by R. Thomson, Wellcome Laboratories) or monoclonal antibodies to glial fibrillary acidic protein (Amersham Corp.) in phosphate-buffered saline containing 2% normal goat serum and 0.2% Triton X-100. After a 24-h incubation at 4°C, the cultures were washed and incubated in phosphate-buffered saline containing anti-tetanus toxin (prepared in rabbits; provided by R. Thomson) or biotinylated rabbit anti-mouse antibodies (Vector). The cultures were then rewashed, incubated for 3 h with fluorescein isothiocyanate-goat anti-rabbit antibodies (Tago Inc.) or red streptavidin (Serotec), washed, dried, mounted, and observed with a fluorescence microscope.

Radiolabeling of cells. Neuronal cultures were maintained in supplemented medium (see above) throughout the labeling period. For $^{32}P_i$ labeling, cells were rinsed three times in phosphate-free DME and labeled for 18 h at 37°C in phosphate-free DME containing 2.5 mCi of $^{32}P_i$ (carrier free; ICN Pharmaceuticals, Inc.). The medium for 208F cells, astrocytes, and 2-day neuronal cultures also contained 10% dialyzed, phosphate-free fetal calf serum. For [^{35}S]methionine labeling, cells were rinsed three times in methionine-free DME and labeled for 18 h at 37°C in DME containing 10% methionine and 180 μCi of [^{35}S]methionine (Amersham) per ml. 208F cells and 2-day neuronal cultures were labeled in methionine-free DME containing 10% undialyzed fetal calf serum.

Protein extractions, immunoprecipitations, and immune complex protein kinase assays. Cells were washed three times in ice-cold Tris-buffered saline, lysed in a modified RIPA buffer (0.15 M NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 10 mM sodium phosphate [pH 7.0], 1% aprotinin, 25 μM leupeptin, 2 mM EDTA, 1 mM dithiothreitol), and clarified at 29,000 × *g* for 1 h at 4°C. The protein concentration was determined by the BCA protein assay (Pierce Chemical Co.), and samples were standardized to 40 to 60 μg of protein. pp60$^{c\text{-}src}$ was immunoprecipitated from the lysates, as described previously (10, 11, 39), by using 10 μl of a 1:500 dilution of ascitic fluid from hybridoma 327 (MAb 327) (43), kindly provided by J. Brugge, State University of New York at Stony Brook, and 8 μl of affinity-purified rabbit anti-mouse immunoglobulin G (Cooper Biomedical, Inc.). Under these conditions, antibody was in excess. Immunoprecipitations of ^{32}P-labeled proteins were done in the presence of 75 μg of RNase A (Sigma) per ml. pp60$^{c\text{-}src}$ was assayed for autophosphorylation and phosphorylation of an exogenous substrate, enolase, by incubation for 30 min at 30°C in kinase buffer containing 30 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham), 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 7.0), 10 mM $MnCl_2$, 10 mM dithiothreitol (10, 11, 26), and 1 μg of acid-treated rabbit muscle enolase (Boehringer Mannheim Biochemicals) (18) per reaction. pp60$^{c\text{-}src}$ from rat fibroblasts (208F) was phosphorylated in vitro by protein kinase C, as previously described (32). Proteins were resolved on 7% SDS-polyacrylamide gels (acrylamide-bisacrylamide, 20:1) and detected by direct autoradiography or by using an intensifying screen (^{32}P) or fluorography (^{35}S) at −70°C with presensitized Kodak XAR film. Excised gel pieces were dissolved in 1.2 ml of 30% H_2O_2–70% perchloric acid (2:1) at 55°C for 12 h. ^{35}S and ^{32}P were quantified by liquid scintillation counting in Aquasol (New England Nuclear Corp.). [^{35}S]methionine-labeled proteins were precipitated by spotting lysates onto glass fiber filters which were then washed with 5% trichloracetic acid, dried, and counted in Aquasol.

Peptide mapping and phosphoamino acid analysis. One-dimensional peptide mapping was done in 12% SDS-polyacrylamide gels (acrylamide-bisacrylamide, 39:1) with 100 ng of *Staphylococcus aureus* V8 protease (Miles Laboratories, Inc.), as previously described (12). For two-dimensional tryptic peptide mapping, pp60$^{c\text{-}src}$ from 2×10^6 208F cells or 3×10^6 to 4×10^6 neurons was separated on SDS-polyacrylamide gels, extracted, oxidized, and digested with trypsin, as described previously (4). Peptides were separated in two dimensions on 100-μm cellulose thin-layer plates by electrophoresis (pH 1.9; 1 kV for 25 min) and chromatography (*n*-butanol–pyridine–acetic acid–H_2O, 75:50:15:60 [by volume]) (38). Phosphoamino acid analyses were done by partially hydrolyzing peptides in 5.7 M HCl at 110°C for 1 h and separating the phosphoamino acids on 100-μm cellulose thin-layer plates by electrophoresis in two dimensions (21).

RESULTS

To study the expression of pp60$^{c\text{-}src}$ in differentiating neurons, we used cultured cells from embryonic rat striatum. Primary cells dissociated from E15 striata were cultured in 7.5% fetal calf serum for 4 days and then in serum-free medium for 14 days. Photomicrographs of the

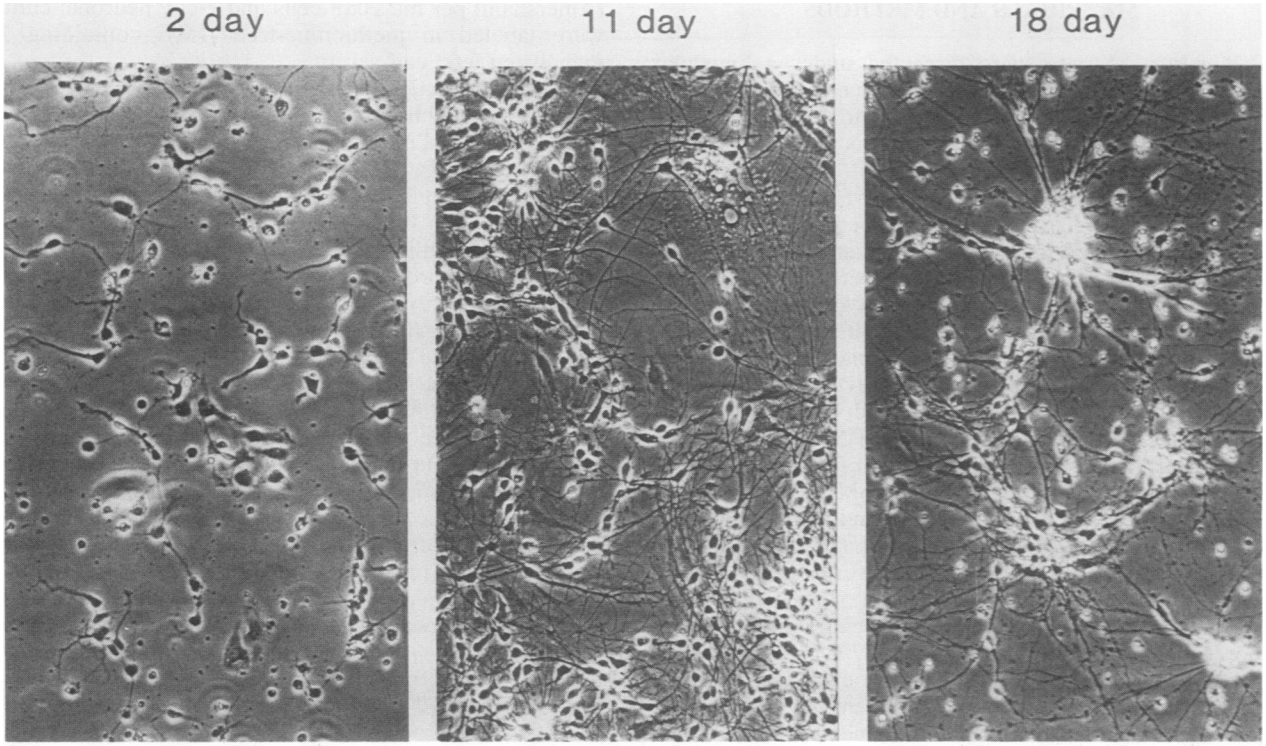

FIG. 1. Effects of serum withdrawal on rat striatal neurons. Cells dissociated from E15 rat striata were maintained in 7.5% fetal calf serum for 4 days and then in serum-free medium for an additional 14 days, as described in Materials and Methods. Photographs were taken of representative phase-contrast microscopic fields at various times after plating (2-, 11-, and 18-day cultures).

cultures at various times after plating are shown in Fig. 1. Cells maintained for up to 18 days in the presence of serum showed limited neurite extension (day 2 shown). In contrast, cells maintained for 7 or 14 days after serum withdrawal (day 11 or 18, respectively) had a dense network of elongated processes characteristic of differentiated neurons. At this time, more than 98% of the cells were found to bind tetanus toxin, whereas less than 2% of the cells bound antibodies to glial fibrillary acidic protein, a specific marker for astrocytes (data not shown). These results show that a relatively pure population of cultured striatal neurons can be induced to differentiate under serum-free conditions.

Two forms of pp60$^{c\text{-}src}$ exist in undifferentiated neurons. To characterize pp60$^{c\text{-}src}$ in undifferentiated neurons, cells were dissociated from E15 striata, cultured for 24 to 30 h in serum, and then incubated for an additional 18 h with [^{35}S]methionine or $^{32}P_i$. Cultures of a rat fibroblast cell line (208F) were labeled in parallel. Two ~60-kDa proteins with equal intensity of labeling were specifically precipitated from lysates of [^{35}S]methionine-labeled undifferentiated neurons (Fig. 2A, lane 2) with MAb 327, a monoclonal antibody specific for pp60$^{c\text{-}src}$, whereas only one 60-kDa protein was precipitated from fibroblasts (lane 1). The faster-migrating of the two neuronal proteins had a mobility similar to that of fibroblast pp60$^{c\text{-}src}$, whereas the slower-migrating form had an apparent molecular mass 1 kDa larger. To determine whether the two neuronal proteins were pp60$^{c\text{-}src}$, *S. aureus* V8 protease mapping was peformed on a gel fragment containing the doublet, which had been rotated counterclockwise through 90 degrees, side-by-side with a gel fragment containing fibroblast pp60$^{c\text{-}src}$. The V8 fragments of the 60-kDa neuronal protein (lane 4, right side) were similar to those of fibroblast pp60$^{c\text{-}src}$ (lane 3), including the 34-kDa V1 fragment, which gives a very weak signal due to the paucity of methionine residues in the NH_2-terminal half of pp60$^{c\text{-}src}$. The 26-kDa V2 fragment of the 61-kDa neuronal protein (lane 4, left side) was similar to that of fibroblast pp60$^{c\text{-}src}$ and that of the 60-kDa neuronal protein. However, the NH_2-terminal V1 fragment of the 61-kDa neuronal protein migrated more slowly than V1 fragments of the other two proteins. We conclude that undifferentiated neurons contain two forms of pp60$^{c\text{-}src}$. One form is similar to pp60$^{c\text{-}src}$ in fibroblasts. The other form, which migrated with an apparent molecular mass 1 kDa larger than that of the lower form, differs in the NH_2-terminal region of the molecule, and this accounts for its retarded electrophoretic mobility.

In most experiments, a single phosphorylated protein was detected in undifferentiated neurons labeled with $^{32}P_i$ (Fig. 2B, lane 2), which comigrated with ^{32}P-labeled pp60$^{c\text{-}src}$ from fibroblasts (lane 1). In some experiments a poorly labeled phosphoprotein comigrating with the upper form of neuronal pp60$^{c\text{-}src}$ was detected (data not shown). The ^{32}P-labeled V8 fragments of neuronal and fibroblast pp60$^{c\text{-}src}$ were similar (Fig. 2B, lanes 3 and 4). When an upper ^{32}P-labeled form of pp60$^{c\text{-}src}$ was detected, faint bands migrating above V1, V3, and V4 were noted upon *S. aureus* V8 digestion (data not shown). Phosphoamino acid analysis identified phosphotyrosine in the COOH-terminal V2 peptide and phosphoserine in the NH_2-terminal V1, V3, and V4 peptides of fibroblast and neuronal pp60$^{c\text{-}src}$ (data not shown). Therefore, the major phosphorylated form of pp60$^{c\text{-}src}$ in undifferentiated neurons is apparently indistinguishable from that in fibroblasts.

Differentiated neurons contain two phosphorylated forms of pp60$^{c\text{-}src}$. To determine whether the expression of pp60$^{c\text{-}src}$ changed upon differentiation of neurons, cells were dissoci-

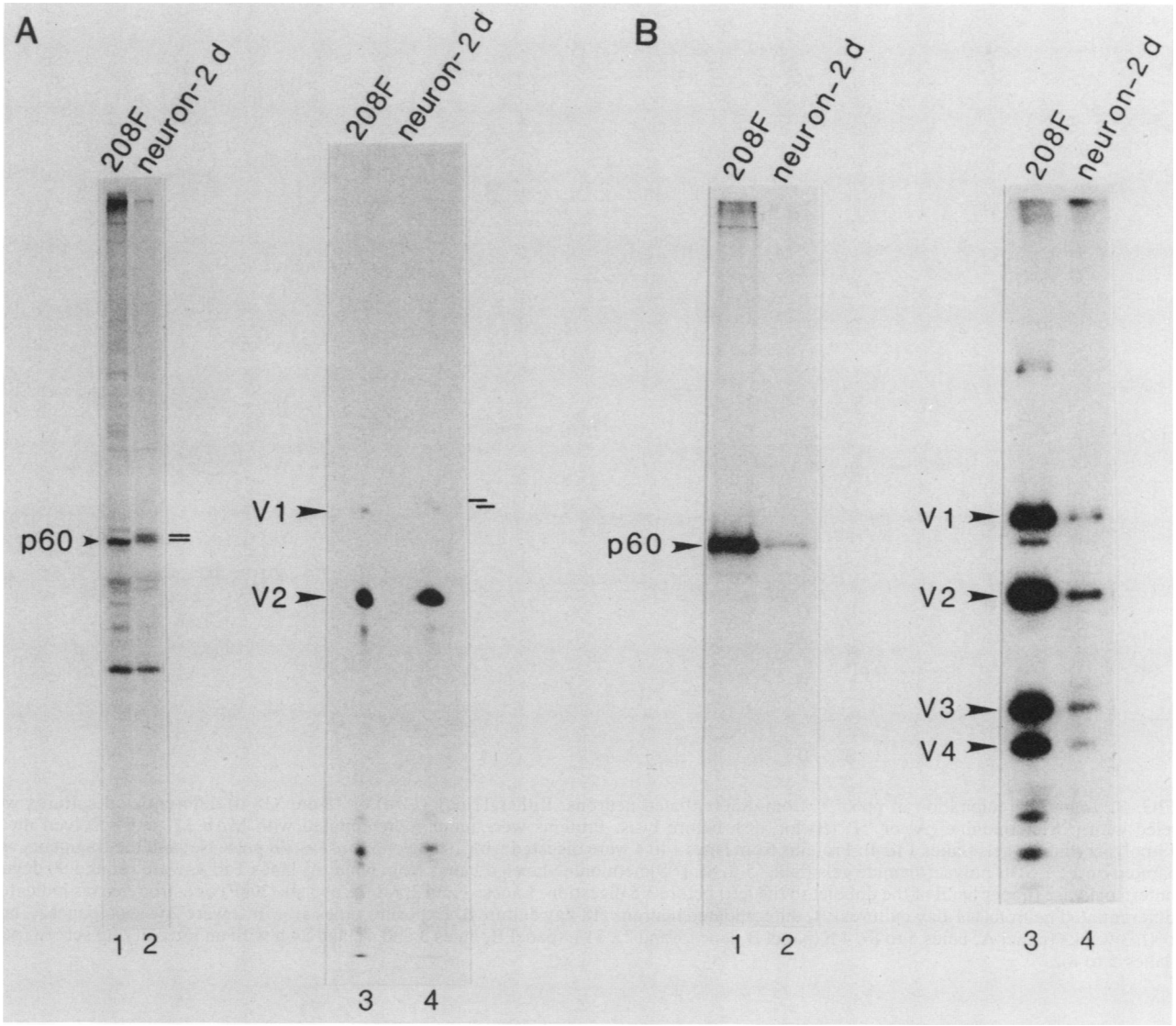

FIG. 2. Immunoprecipitation of pp60$^{c\text{-}src}$ from undifferentiated neurons. One-day cultures of striatal neurons were labeled with [^{35}S]methionine (A) or $^{32}P_i$ (B) for an additional 18 h before lysis. Proteins were immunoprecipitated with MAb 327 and resolved on 7% SDS-polyacrylamide gels (lanes 1 and 2). Proteins from lanes 1 and 2 were digested with 100 ng of *S. aureus* V8 protease, and the fragments were resolved on 12% SDS-polyacrylamide gels (lanes 3 and 4). The [^{35}S]methionine-labeled bands from panel A, lanes 1 and 2 were rotated 90 degrees counterclockwise (upper band of the doublet to the left) before V8 digestion. V8 fragments: V1, 34-kDa NH_2-terminal fragment (doublet indicated by horizontal bars to the right of panel A, lane 4); V2, 26-kDa COOH-terminal fragment; V3 and V4, 18- and 16-kDa NH_2-terminal fragments, respectively, derived from further cleavage of V1. Lanes: 1 and 3, rat fibroblasts (208F); 2 and 4, undifferentiated neurons (2-day cultures). Exposure times at −70°C were 2 weeks (panel A, lanes 1 and 2), 3 weeks (panel A, lanes 3 and 4), 23 h (panel B, lanes 1 and 2), and 35 h with an intensifying screen (panel B, lanes 3 and 4).

ated from E15 striata and cultured for 4 days in serum then for 7 or 14 days in serum-free medium before being incubated for 18 h with [^{35}S]methionine or ^{32}Pi. To compare pp60$^{c\text{-}src}$ from neurons and interstitial cells, astrocytes were prepared from whole brains of 2-day-old rats, cultured in 7.5% serum for 15 days, and then incubated for 18 h with $^{32}P_i$. Two ~60-kDa [^{35}S]methionine-labeled proteins were specifically immunoprecipitated by MAb 327 from lysates of differentiated neurons (Fig. 3A, lanes 3 and 4), whereas a single 60-kDa protein was precipitated from fibroblasts (lane 2) or astrocytes (lane 1). As with undifferentiated neurons, the faster-migrating differentiated neuronal protein had a mobility similar to that of pp60$^{c\text{-}src}$ from fibroblasts or astrocytes, and the slower-migrating form had an apparent molecular mass 1 kDa larger. The V8 fragments of pp60$^{c\text{-}src}$ from fibroblasts and astrocytes and the 60-kDa neuronal protein were similar (lanes 5, 6, 7 [right], and 8 [right]). *S. aureus* V8 digestion of the 61-kDa neuronal protein (lanes 7 [left] and 8 [left]) showed that the COOH-terminal V2 fragment was identical to that of fibroblast or astrocyte pp60$^{c\text{-}src}$, whereas the NH_2-terminal V1 fragment migrated more slowly than the corresponding V1 fragments of the other proteins. Therefore, the two [^{35}S]methionine-labeled proteins in the differentiated neurons were similar to those in the undifferentiated cells, except that the upper form often predominated in immunoprecipitates from differentiated cells (e.g., lane 3).

Two ~60-kDa ^{32}P-labeled proteins were immunoprecipi-

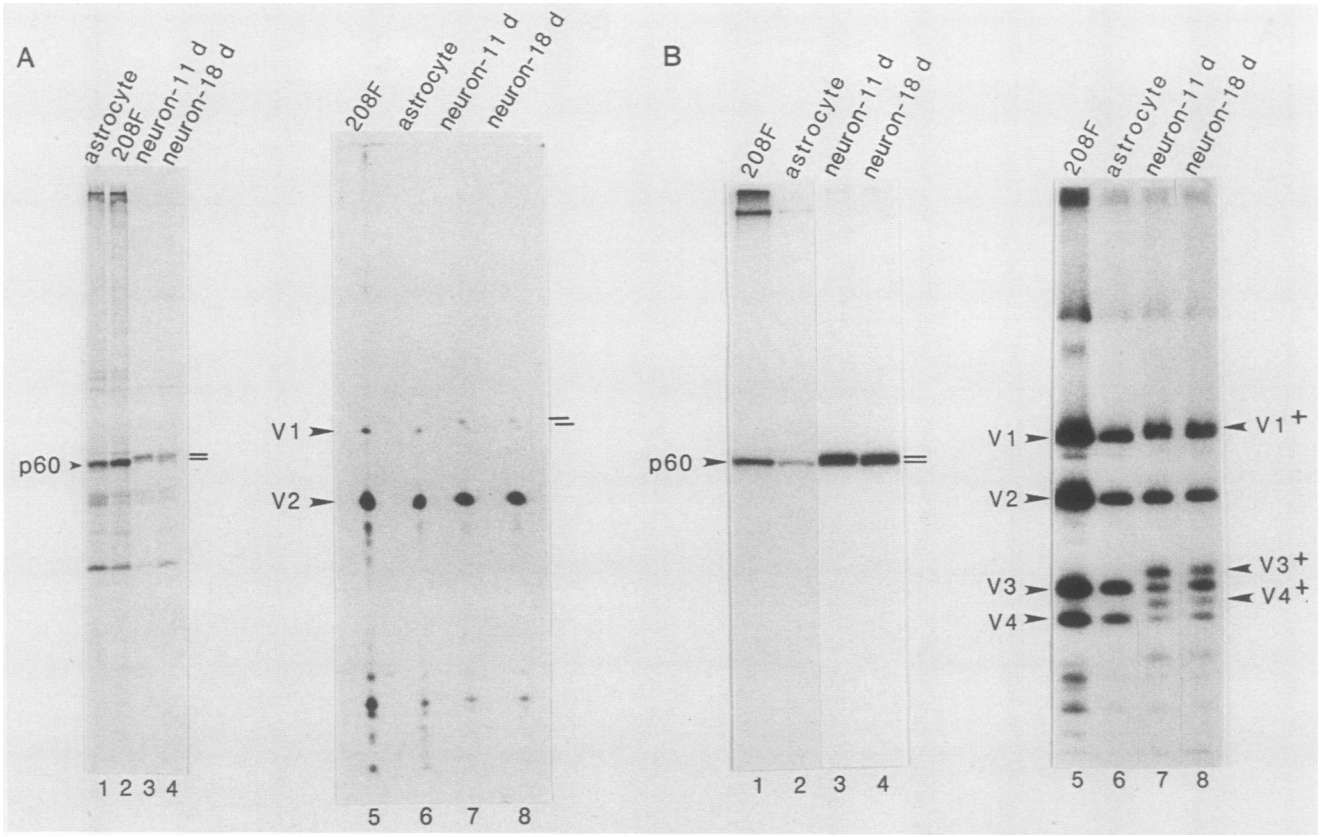

FIG. 3. Immunoprecipitation of pp60$^{c\text{-}src}$ from differentiated neurons. Either 11-day (11 d) or 18-day (18 d) differentiated cultures were labeled with [^{35}S]methionine (A) or $^{32}P_i$ (B) for 18 h before lysis. Proteins were immunoprecipitated with MAb 327 and resolved on 7% SDS-polyacrylamide gels (lanes 1 to 4). Proteins from lanes 1 to 4 were digested with 100 ng of *S. aureus* V8 protease, and the fragments were separated on 12% SDS-polyacrylamide gels (lanes 5 to 8). [^{35}S]methionine-labeled bands from panel A, lanes 1 to 4, were rotated 90 degrees counterclockwise (upper band of the doublet to the left) before V8 digestion. Lanes: 1 and 2, rat fibroblasts (208F) or astrocytes (as indicated); 3, differentiated neurons (11-day cultures); 4, differentiated neurons (18-day cultures). Exposure times at −70°C were 2 weeks (panel A, lanes 1 to 4), 3 weeks (panel A, lanes 5 to 8), 4 h (panel B, lanes 1 and 2), 31 h (panel B, lanes 3 and 4), and 24 h with an intensifying screen (panel B, lanes 5 to 8).

tated from lysates of differentiated neurons (Fig. 3B, lanes 3 and 4). The faster-migrating protein had a mobility similar to that of ^{32}P-labeled pp60$^{c\text{-}src}$ from fibroblasts (lane 1) or astrocytes (lane 2). Further analysis by one-dimensional V8 protease mapping showed that the V8 fragments of astrocyte pp60$^{c\text{-}src}$ (lane 6) were identical to those of fibroblast pp60$^{c\text{-}src}$ (lane 5). However, the NH_2-terminal V1, V3, and V4 fragments of pp60$^{c\text{-}src}$ from differentiated neurons (lanes 7 and 8) all appeared as doublets (the upper band of each doublet is designated V1$^+$, V3$^+$, and V4$^+$, respectively). The lower band of each doublet had a mobility similar to that of the corresponding fibroblast and astrocyte V8 fragments. The relative intensities of the members of each doublet correlated well with those of the upper and lower forms of the original pp60$^{c\text{-}src}$ doublet. Phosphotyrosine was the only phosphoamino acid in the COOH-terminal V2 fragment, and phosphoserine was the only phosphoamino acid in the NH_2-terminal V1, V3, and V4 fragments of each protein (data not shown). The additional V1$^+$, V3$^+$, and V4$^+$ fragments of pp60$^{c\text{-}src}$ from differentiated neurons were phosphorylated exclusively on serine. These results show that differentiated neurons contain two phosphorylated forms of pp60$^{c\text{-}src}$. The 60-kDa form appears similar to pp60$^{c\text{-}src}$ in fibroblasts. The 61-kDa form differs from the fibroblast form in the NH_2-terminal 18-kDa region of the molecule.

To determine whether neuronal pp60$^{c\text{-}src}$ had novel sites of phosphorylation, we compared the tryptic phosphopeptides of pp60$^{c\text{-}src}$ from differentiated neurons with those of pp60$^{c\text{-}src}$ from rat fibroblasts (Fig. 4). Tryptic digestion of ^{32}P-labeled fibroblast pp60$^{c\text{-}src}$ generated five major phosphopeptides (Fig. 4A). Peptides 1 and 2 are known to be phosphorylated on Tyr-527 (19), peptide 5 is phosphorylated on Ser-17 (16, 40, 50), and peptides 3 and 4 are phosphorylated on unidentified NH_2-terminal serines (11). Tryptic digestion of pp60$^{c\text{-}src}$ from differentiated neurons (the upper and lower phosphoproteins in the doublet combined) generated seven major peptides (Fig. 4B). Peptides 1 and 2 contained phosphotyrosine, and peptides 3 to 7 contained phosphoserine (data not shown; Fig. 4F). A mixture of tryptic peptides of pp60$^{c\text{-}src}$ from fibroblasts and differentiated neurons (Fig. 4C) confirmed that peptides 1 to 5 of the two proteins were identical. In no instance did we observe a phosphopeptide containing phosphorylated Tyr-416. Therefore, pp60$^{c\text{-}src}$ from differentiated neurons had the same phosphorylation sites as did fibroblast pp60$^{c\text{-}src}$ and, in addition, was phosphorylated on serines contained in two other tryptic peptides. One of these (peptide 6) was similar to the tryptic

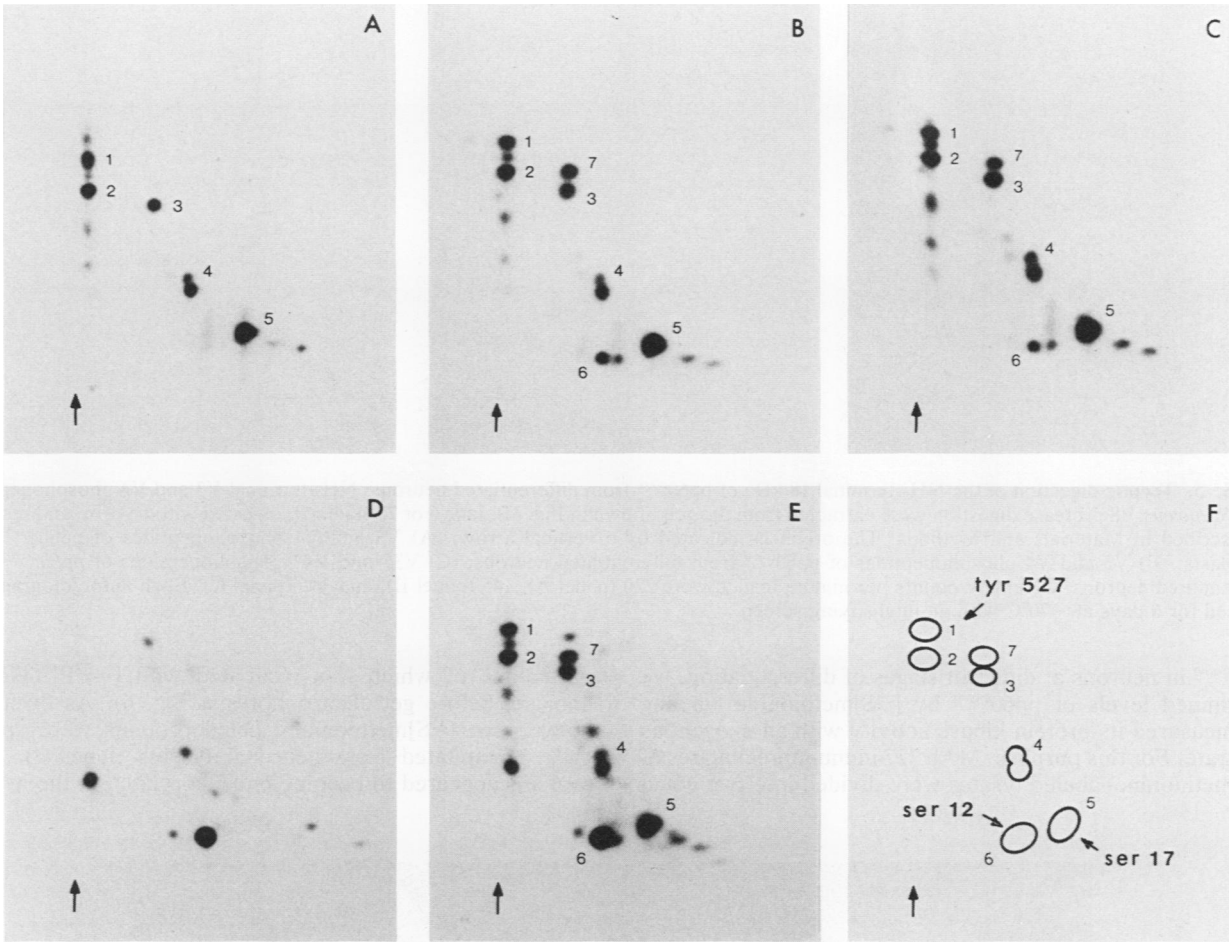

FIG. 4. Tryptic digestion of pp60$^{c\text{-}src}$ from differentiated neurons. Phosphoproteins were extracted from the gels shown in Fig. 3B, lane 1 or 3, oxidized, digested with trypsin, and resolved by electrophoresis at pH 1.9 in the horizontal dimension (anode on left) and chromatography in the vertical dimension, as described in Materials and Methods. The origin is indicated by a vertical arrow. (A) pp60$^{c\text{-}src}$ from rat fibroblasts; (B) pp60$^{c\text{-}src}$ from differentiated neurons (11-day cultures); (C) mixture of phosphopeptides from panels A and B; (D) rat fibroblast pp60$^{c\text{-}src}$ phosphorylated in vitro by protein kinase C; (E) mixture of phosphopeptides from panels B and D; (F) schematic of pp60$^{c\text{-}src}$ phosphopeptides and their phosphoamino acid composition. Cerenkov counts per minute were 420 (panel A), 450 (panel B), 420 of A and 450 of B (panel C), 425 (panel D), and 450 of B and 425 of D (panel E). Each autoradiogram was exposed for 5 days at −70°C with an intensifying screen.

peptide of pp60$^{c\text{-}src}$ phosphorylated in vitro on Ser-12 by protein kinase C (32) (Fig. 4D). A mixture of these peptides confirmed that they were identical (Fig. 4E). Therefore, one of the serines phosphorylated in pp60$^{c\text{-}src}$ from differentiated neurons and not in pp60$^{c\text{-}src}$ from a fibroblast cell line is Ser-12.

To determine whether the additional serine phosphorylations were found in one or both forms of pp60$^{c\text{-}src}$ from differentiated neurons, we analyzed individual ^{32}P-labeled pp60$^{c\text{-}src}$ V8 fragments (Fig. 3) by tryptic peptide mapping (Fig. 5). Tryptic digestion of the COOH-terminal V2 peptide generated two phosphopeptides which appeared identical to peptides 1 and 2 in whole-protein digests (data not shown). A tryptic digest of the fibroblast pp60$^{c\text{-}src}$ NH_2-terminal V3 and V4 peptides combined contained three major phosphopeptides (Fig. 5A), which appeared identical to peptides 3, 4, and 5 from digest of whole fibroblast protein (Fig. 4A). The digest of the NH_2-terminal fragments of the lower band of neuronal pp60$^{c\text{-}src}$ (V3 and V4 peptides combined) (Fig. 5B) also contained three major phosphopeptides, which were similar to those of fibroblast pp60$^{c\text{-}src}$ (Fig. 5A) and to peptides 3, 4, and 5 of whole neuronal pp60$^{c\text{-}src}$ (Fig. 4B). Therefore, the phosphorylated protein present in the lower band of the doublet of neuronal pp60$^{c\text{-}src}$ is similar, as determined by both tryptic and V8 phosphopeptide mapping, to fibroblast pp60$^{c\text{-}src}$. A tryptic digest of the NH_2-terminal fragments of the upper band of neuronal pp60$^{c\text{-}src}$ (V3$^+$ and V4+ peptides combined) contained five phosphopeptides (Fig. 5C), three of which (peptides 3, 4, and 5) seemed to be identical to those found in the lower band of the doublet (Fig. 5B) and in the NH_2 terminus of fibroblast pp60$^{c\text{-}src}$ (Fig. 5A). The two additional phosphopeptides appeared to be the same as peptides 6 and 7 from digests of whole pp60$^{c\text{-}src}$ from differentiated neurons (Fig. 4B). Therefore, the additional serine phosphorylations found in pp60$^{c\text{-}src}$ from differentiated neurons are contained in the upper phosphorylated species.

pp60$^{c\text{-}src}$ protein kinase was activated in striatal neurons as they differentiated. To determine whether there were any changes in the absolute level or protein kinase activity of

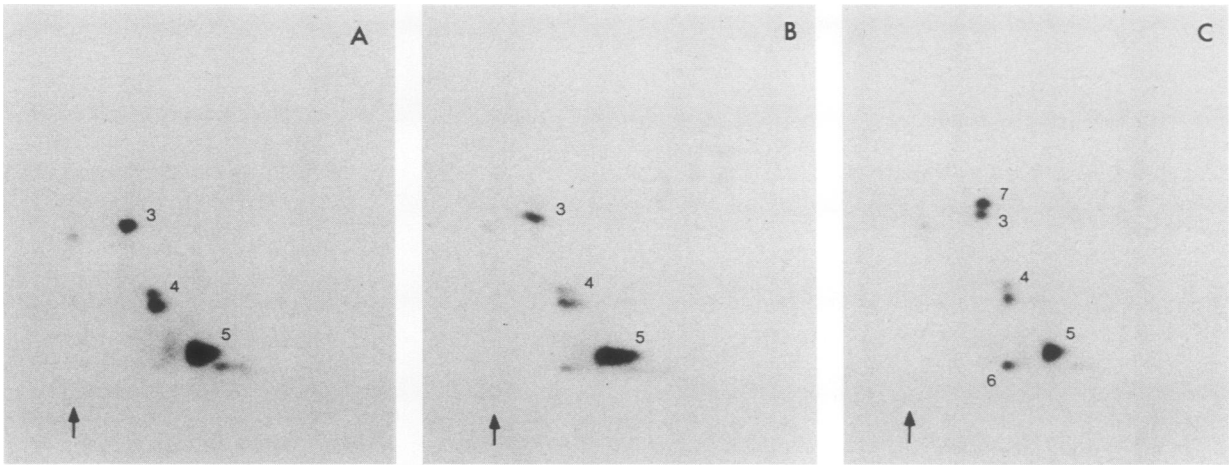

FIG. 5. Tryptic digestion of the NH_2-terminal 18 kDa of $pp60^{c\text{-}src}$ from differentiated neurons. NH_2-terminal V3 and V4 phosphopeptides from *S. aureus* V8 protease digestion were extracted from the gels shown in Fig. 3B, lane 5 or 7, oxidized, digested with trypsin, and resolved as described in Materials and Methods. The origin is indicated by a vertical arrow. (A) V3 and V4 phosphopeptides of $pp60^{c\text{-}src}$ from fibroblasts; (B) V3 and V4 phosphopeptides of $pp60^{c\text{-}src}$ from differentiated neurons; (C) $V3^+$ and $V4^+$ phosphopeptides of $pp60^{c\text{-}src}$ from differentiated neurons. Cerenkov counts per minute loaded were 220 (panel A), 145 (panel B), and 115 (panel C). Each autoradiogram was exposed for 6 days at −70°C with an intensifying screen.

$pp60^{c\text{-}src}$ in neurons at different stages of differentiation, we determined levels of $pp60^{c\text{-}src}$ by [^{35}S]methionine labeling and measured its protein kinase activity with an exogenous substrate. For this purpose, MAb 327 immunoprecipitates of [^{35}S]methionine-labeled lysates were divided into two equal parts, one of which was incubated with [γ-^{32}P]ATP and enolase, before gel electrophoresis (Fig. 6). As discussed above, two [^{35}S]methionine-labeled proteins were specifically precipitated from neuronal lysates (lanes 2). Both proteins appeared to become phosphorylated in the in vitro

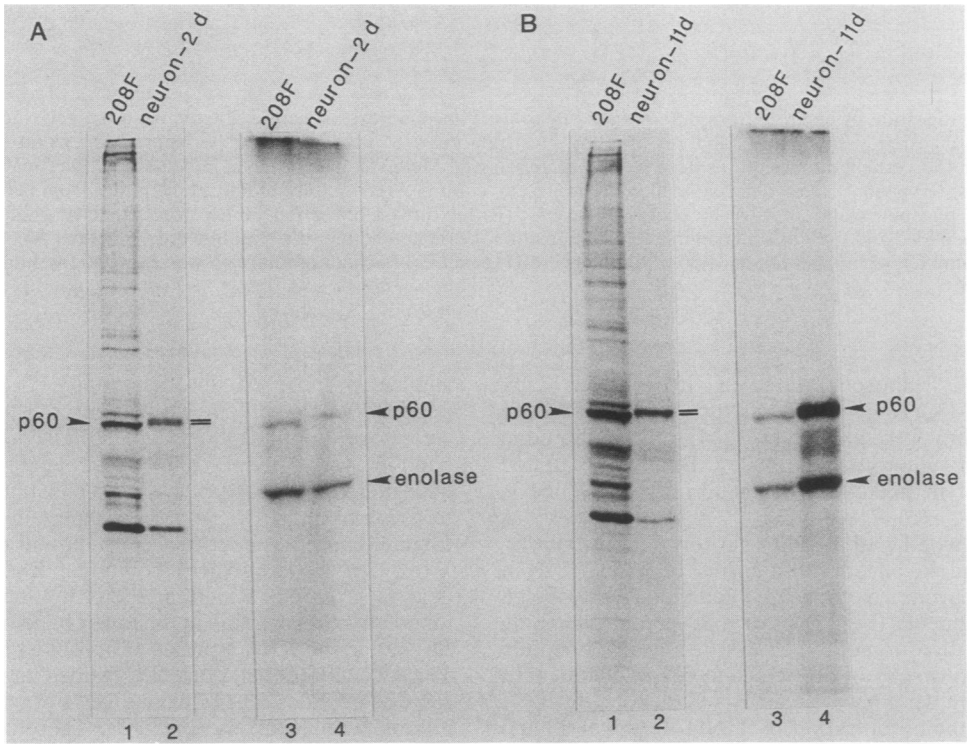

FIG. 6. Specific activity of $pp60^{c\text{-}src}$ protein kinase from striatal neurons. Either 1-day (A) or 11-day (B) cultures were labeled with [^{35}S]methionine for an additional 18 h before lysis. MAb 327 immunoprecipitates were divided into two equal parts, one of which was phosphorylated in vitro with [γ-^{32}P]ATP in the presence of enolase (lanes 3 and 4). Proteins were resolved on 7% SDS-polyacrylamide gels. Lanes: 1 and 3, rat fibroblasts (208F); 2 and 4, undifferentiated neurons (2-day cultures) (panel A) or differentiated neurons (11-day cultures) (panel B). Exposure times at −70°C were 24 h (panel A, lanes 1 and 2), 48 h (panel A, lanes 3 and 4) (gel covered with aluminum foil to block [^{35}S]methionine), 48 h (panel B, lanes 1 and 2), and 2 h with an intensifying screen (panel B, lanes 3 and 4).

TABLE 1. Specific activity of pp60$^{c\text{-}src}$ protein kinase in striatal neurons[a]

Cells	pp60$^{c\text{-}src}$/total protein (pg/μg) (relative level)	Autophosphorylation		Enolase phosphorylation	
		^{32}P cpm/ng of pp60$^{c\text{-}src}$	Relative sp act	^{32}P cpm/ng of pp60$^{c\text{-}src}$	Relative sp act
208F	38 (1.3)	115	1.1	109	1.1
Neurons (2-day cultures)	29 (1.0)	106	1.0	101	1.0
Neurons (11-day cultures)	34 (1.2)	1,525	14.4	685	6.8

[a] The amount of pp60$^{c\text{-}src}$ in immunoprecipitates of [^{35}S]methionine-labeled 208F lysates was calculated by excising gel pieces containing the protein, determining the incorporated counts per minute, and dividing that value by the trichloroacetic acid-precipitable [^{35}S]methionine counts per minute per microgram of lysate protein. It was assumed that [^{35}S]methionine incorporation into pp60$^{c\text{-}src}$ was similar to [^{35}S]methionine incorporation into total cell protein. ^{32}P incorporation into protein was determined by counting the radioactivity in excised gel pieces. The relative values for the neuronal samples were determined by scanning densitometry and are expressed as picograms of pp60$^{c\text{-}src}$ per microgram of total cell protein (^{35}S) or counts per minute per nanogram of pp60$^{c\text{-}src}$ (^{32}P) based on the counts per minute incorporated into fibroblast pp60$^{c\text{-}src}$. Each value represents the mean of duplicate samples, which varied less than 10%. The analysis is from the experiment shown in Fig. 6. Scanning densitometry from two additional experiments gave similar results.

kinase reaction (lanes 4). The amount of pp60$^{c\text{-}src}$ in the immunoprecipitates and ^{32}P incorporation into pp60$^{c\text{-}src}$ and enolase were calculated as described in Materials and Methods and Table 1, footnote *a*. Trichloroacetic acid-precipitable [^{35}S]methionine counts per minute per microgram of lysate protein was used as a measure of incorporation into protein. The value for fibroblasts was not significantly different from that for undifferentiated neurons but was approximately twofold higher than that for differentiated neurons. The abundance of pp60$^{c\text{-}src}$ is expressed as picograms of pp60$^{c\text{-}src}$ per microgram of total cell protein, and its specific protein kinase activity is given as ^{32}P counts per minute per nanogram of total pp60$^{c\text{-}src}$ (Table 1). Both values are also shown normalized to the value obtained for undifferentiated neurons (Table 1).

The amount of pp60$^{c\text{-}src}$ in 208F cells (0.004% of total cell protein) was close to the estimate made by others of the amount of pp60$^{c\text{-}src}$ in chick fibroblasts (14), as well as our own measurement of pp60$^{c\text{-}src}$ in mouse NIH 3T3 fibroblasts. The level of pp60$^{c\text{-}src}$ in undifferentiated striatal neurons was similar to that in 208F fibroblasts and astrocytes (data not shown) and only increased slightly upon differentiation. The specific protein kinase activity of pp60$^{c\text{-}src}$ from undifferentiated neurons was similar to that from fibroblasts or astrocytes (data not shown). In contrast, the specific activity of pp60$^{c\text{-}src}$ from differentiated neurons was 14 or 7 times that of pp60$^{c\text{-}src}$ from undifferentiated neurons, as measured by autophosphorylation or phosphorylation of enolase, respectively. Similar data were obtained with pp60$^{c\text{-}src}$ isolated from neurons allowed to differentiate for 7 or 14 days (11- or 18-day cultures, respectively) (data not shown).

DISCUSSION

Two major findings emerged from our study of pp60$^{c\text{-}src}$ in rat embryonic striatal neurons. First, in contrast to fibroblasts and astrocytes, two forms of pp60$^{c\text{-}src}$ were present in both undifferentiated and differentiated rat embryonic striatal neurons. One form is similar to pp60$^{c\text{-}src}$ in fibroblasts, whereas the other has an apparent molecular mass 1 kDa larger due to a difference in the NH_2-terminal 18 kDa of the protein. Second, during neuronal differentiation, the specific protein kinase activity of pp60$^{c\text{-}src}$ increased. These results raise a number of important issues. Is the neuron-specific 61-kDa protein immunoprecipitated from [^{35}S]methionine-labeled cultures of neurons derived from the c-*src* gene or from a closely related gene? If it is expressed from the c-*src* gene, does it differ in primary structure or as a result of posttranslational modification from the 60-kDa form? Is the 60-kDa form of pp60$^{c\text{-}src}$ present in neurons identical to pp60$^{c\text{-}src}$ from fibroblasts? Does the 61-kDa phosphorylated protein detected in differentiated neurons correspond to the [^{35}S]methionine-labeled 61-kDa protein? By what mechanism is the protein kinase activity of pp60$^{c\text{-}src}$ increased in differentiated neurons, and is the increase related to the presence of the neuron-specific form of pp60$^{c\text{-}src}$?

The neuron-specific 61-kDa protein immunoprecipitated by a monoclonal antibody specific for pp60$^{c\text{-}src}$ is closely related to pp60$^{c\text{-}src}$ structurally and gave rise to a similar pattern of V8 cleavage products which had the same relative methionine and phosphate contents. The phosphorylated 61-kDa protein has three NH_2-terminal tryptic phosphopeptides in common with rat fibroblast pp60$^{c\text{-}src}$, including the phosphopeptide containing Ser-17. An additional peptide of the 61-kDa protein comigrated with a pp60$^{c\text{-}src}$ peptide known to contain Ser-12. Only a single c-*src* gene has been detected in mammals, and none of the pp60$^{c\text{-}src}$-related genes, which have been sequenced at the NH_2 terminus (*syn*, c-*yes*, *lyn*, and *lck*), has a sequence that could give rise to tryptic peptides equivalent to those from pp60$^{c\text{-}src}$ containing Ser-12 or Ser-17 (47, 60, 65, 67, 71). Therefore, the phosphorylated 61-kDa protein, which appeared upon differentiation, is likely to be encoded by the c-*src* gene rather than by a closely-related gene. If, as seems probable (see below), the phosphorylated 61-kDa protein is derived from the 61-kDa protein detected by [^{35}S]methionine labeling, then we can conclude that the latter protein is also a form of pp60$^{c\text{-}src}$. By most criteria, the neuronal 60-kDa protein is indistinguishable from fibroblast pp60$^{c\text{-}src}$, but formal proof is lacking that they are identical and we noted that this protein sometimes migrated a little slower than fibroblast pp60$^{c\text{-}src}$.

If the neuron-specific 61-kDa protein is derived from the c-*src* gene, then it could differ from fibroblast pp60$^{c\text{-}src}$ either as a result of posttranslational modification or in primary structure. With regard to the former possibility, the NH_2-terminal difference might result from the addition of phosphate itself, since there are precedents for decreased SDS gel mobility dependent on phosphorylation. However, this seems unlikely for several reasons. First, although the phosphorylated 61-kDa protein in neurons is phosphorylated at additional serines, these sites were not phosphorylated to the same stoichiometry as Ser-17 (Fig. 4), indicating that not all the pp60$^{c\text{-}src}$ molecules in this band were phosphorylated at these additional sites. Second, although phosphorylation of Ser-12 (32) or of unidentified NH_2-terminal tyrosines (10, 13, 72) can cause small decreases in the mobility of fibroblast pp60$^{c\text{-}src}$, there is no known in vivo phosphorylation event which results in such a dramatic shift in the mobility of pp60$^{c\text{-}src}$. Third, the 61-kDa protein does not appear to be

more highly phosphorylated than the 60-kDa protein, since the $^{32}P/^{35}S$ ratio for each protein was similar (Fig. 3, lanes 3 and 4). Fourth, there is no change in the mobility of [^{35}S]methionine-labeled neuron-specific pp60$^{c\text{-}src}$ after treatment with potato acid phosphate (C. A. Cartwright, unpublished results). Fifth, there was a significant level of the neuron-specific form of pp60$^{c\text{-}src}$ in undifferentiated neurons, yet it apparently had a very low phosphate content. Sixth, two-dimensional gel analysis of the two pp60$^{c\text{-}src}$ species indicates that the neuron-specific form is more basic than the fibroblast form (K. L. Gould and C. A. Cartwright, unpublished results), which is clearly inconsistent with the neuronal form being derived by phosphorylation from the fibroblast form. Another type of posttranslational modification might be responsible for the NH_2-terminal alteration, but most of the recognized modifications, like phosphorylation, make proteins more acidic.

A possible explanation for the neuron-specific form of pp60$^{c\text{-}src}$ is that it differs in primary sequence from the fibroblast form somewhere in the NH_2-terminal 18 kDa. Such a difference could result from the use of alternate splicing pathways for a single c-*src* gene transcript, as is the case for mouse c-*abl* (5) and *Drosophila* epidermal growth factor receptor mRNAs (56), which have multiple different 5′-coding exons. The fact that there are precedents for alternative splicing pathways in neuronal cells (54) lends credence to this idea. The isolation of cDNA clones for pp60$^{c\text{-}src}$ from neuronal cells may help resolve this question.

The neuron-specific form of pp60$^{c\text{-}src}$ appeared to be poorly phosphorylated in undifferentiated neurons. The reason for this is unclear. Since we were able to isolate a phosphorylated protein with the same mobility from differentiated neurons, it is unlikely that phosphate was being lost during immunoprecipitation. It is possible that the 61-kDa protein is not phosphorylated in differentiated neurons and that the more slowly migrating phosphorylated form of pp60$^{c\text{-}src}$ is derived from the 60-kDa form of pp60$^{c\text{-}src}$ by additional phosphorylation, which causes a fortuitous comigration. However, as argued above, it seems unlikely that phosphorylation could result in such a mobility shift. In addition, the amount of ^{32}P label in the 61-kDa protein was proportional to the amount of [^{35}S]methionine label in this protein in differentiated neurons (Fig. 3). This supports the idea that the phosphorylated 61-kDa protein is derived from the neuron-specific form of pp60$^{c\text{-}src}$ and that phosphorylation of the 61-kDa protein is in some way regulated during differentiation.

The increase in specific activity of pp60$^{c\text{-}src}$ during neuronal differentiation correlated with the appearance of the phosphorylated form of the neuron-specific form of pp60$^{c\text{-}src}$. Since we could not measure the protein kinase activity of the two forms of pp60$^{c\text{-}src}$ separately, we do not know whether both were activated. In principle, the extent of autophosphorylation of each species could be determined individually, but this is not an ideal measure of protein kinase activity toward exogenous substrates. A priori, the mechanism of activation could be due to a posttranslational modification, of which the most likely is phosphorylation, or could result from a different primary structure. The NH_2-terminal region of pp60^{src} has been implicated in the regulation of its enzymatic activity, since pp60$^{v\text{-}src}$ (8, 13) and pp60$^{c\text{-}src}$ (6, 10, 51, 72) phosphorylated on NH_2-terminal tyrosine residues have elevated protein kinase activity and a number of temperature-sensitive mutations affecting protein kinase activity map to this region (37). This suggests that the additional sites of serine phosphorylation in the NH_2 terminus of neuron-specific pp60$^{c\text{-}src}$ could be pertinent to activation of neuronal pp60$^{c\text{-}src}$. However, one of these sites is Ser-12, and we have previously found that phosphorylation of Ser-12 had no detectable stimulatory effect on the protein kinase activity of fibroblast pp60$^{c\text{-}src}$. Phosphorylation of the other unidentified serine could be a cause of activation of the pp60$^{c\text{-}src}$ kinase. However, there are no reported instances in which activity of a protein-tyrosine kinase is increased by serine phosphorylation, even though the activity of protein-serine kinases can be regulated in this manner (41).

The protein kinase activity of fibroblast pp60$^{c\text{-}src}$ is regulated in a negative fashion by phosphorylation of Tyr-527 on pp60$^{c\text{-}src}$ (20, 23). This may explain why pp60$^{c\text{-}src}$, which is associated with polyomavirus middle tumor antigen and lacks phosphate on Tyr-527, has increased protein kinase activity (7, 10, 11, 23, 25). However, the activation of neuronal pp60$^{c\text{-}src}$ is apparently not due to a lack of Tyr-527 phosphorylation, since this residue seems to be phosphorylated to the same extent in neuronal pp60$^{c\text{-}src}$ as in fibroblast pp60$^{c\text{-}src}$. In addition, the activation of the middle tumor antigen-associated pp60$^{c\text{-}src}$ is accompanied by phosphorylation on Tyr-416, the major autophosphorylation site, which is not the case for activated neuronal pp60$^{c\text{-}src}$. The lack of detectable Tyr-416 phosphorylation in the activated neuronal pp60$^{c\text{-}src}$ is particularly interesting because it violates an otherwise perfect correlation with enzymatic activation of pp60^{src}.

Other investigators have reported structural and functional alterations of pp60$^{c\text{-}src}$ in cells derived from nervous tissue. Rosen et al. (53) found high levels of pp60$^{c\text{-}src}$ protein kinase activity in neural cell lines and tumors of neuroectodermal origin. Bolen et al. (6) showed that one human neuroblastoma cell line, KCNR, contains a distinct form of pp60$^{c\text{-}src}$ which is phosphorylated on tyrosine in its NH_2-terminal region. Brugge et al. (9) reported that activated pp60$^{c\text{-}src}$ in differentiated neurons form rat embryos has an alteration within the NH_2-terminal region of the molecule that retards its electrophoretic mobility. The phosphorylated neuron-specific pp60$^{c\text{-}src}$ which we report here is similar to the altered form of pp60$^{c\text{-}src}$ found in differentiated rat embryo neurons (9) in that both are phosphorylated on an NH_2-terminal serine(s) not usually phosphorylated in fibroblast or astrocyte pp60$^{c\text{-}src}$ (J. S. Brugge, personal communication).

Two recent studies support our finding that alterations in pp60$^{c\text{-}src}$ accompany neuronal differentiation. Differentiation of an embryonal carcinoma cell line (p19S1801A1) into neuronlike cells resulted in a shift in the mobility of pp60$^{c\text{-}src}$. The shift was due to a difference in the NH_2-terminal region of pp60$^{c\text{-}src}$ (44) similar to the one previously observed in primary cultures of rat neurons (9). Differentiation was also accompanied by an 8- to 20-fold increase both in the amount of pp60$^{c\text{-}src}$ protein and in pp60$^{c\text{-}src}$ protein kinase activity. Our results are in basic agreement with those of Brugge and co-workers in that we detected the neuron-specific form of pp60$^{c\text{-}src}$ and an increase in total pp60$^{c\text{-}src}$ protein kinase activity during differentiation but differ in that we did not find a higher level of pp60$^{c\text{-}src}$ in differentiated or undifferentiated neurons than in fibroblasts. The reason for this discrepancy is not clear but is being investigated by using immunoblotting to provide a more accurate measure of pp60$^{c\text{-}src}$ levels than is provided by metabolic labeling.

It is tempting to speculate that the increase in pp60$^{c\text{-}src}$ protein kinase activity and the expression of the neuron-specific form of pp60$^{c\text{-}src}$ are essential for the differentiation or function of neurons or both. Development of the rat

striatum is accompanied by a three- to fivefold increase in pp60$^{c\text{-}src}$ protein kinase activity between days 15 and 20 of embryonic life (the peak period of neuronogenesis) (C. A. Cartwright and R. Simantov, unpublished data), which is consistent with our data on cultured neurons. An increase in pp60$^{c\text{-}src}$ protein kinase activity has also been observed in nonneuronal cell lines induced to differentiate under a variety of conditions (2, 30), but no new species of pp60$^{c\text{-}src}$ was detected. Thus, although the increase in pp60$^{c\text{-}src}$ protein kinase activity may be important for neuronal differentiation, it may be independent of the existence of the neuron-specific form of pp60$^{c\text{-}src}$. The neuron-specific form of pp60$^{c\text{-}src}$ has been observed by in vitro protein kinase assays and metabolic labeling of tissues from rat brain (C. A. Cartwright and R. Simantov, unpublished data). These results support the concept that neuron-specific pp60$^{c\text{-}src}$ has a functional role in brain neurons.

The notion that pp60$^{c\text{-}src}$ might be involved in neuronal differentiation is supported by the observation that pp60$^{v\text{-}src}$ causes PC12 pheochromocytoma cells to differentiate (1). Treatment of PC12 cells with nerve growth factor also induces differentiation (34). The correlation of neurite outgrowth with increased intracellular cAMP and mobilization of intracellular Ca^{2+} stores after treatment of PC12 cells with nerve growth factor (57) raises intriguing questions about a possible role of pp60$^{c\text{-}src}$ in signal transduction and activation of intracellular second messengers.

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