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c-*src* gene product in developing rat brain is enriched in nerve growth cone membranes

(protooncogene/neuronal differentiation/protein-tyrosine kinase)

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ABSTRACT **Differentiating rat neurons express high levels of the protooncogene product pp60$^{c\text{-}src}$, a 60-kDa tyrosine kinase of unknown function encoded by c-*src*. pp60$^{c\text{-}src}$ was found to be concentrated at least 9-fold in membranes from a subcellular fraction of nerve growth cones, the motile tips of outgrowing neuronal processes. Indirect immunofluorescence staining of cultured chick retinal explants showed pp60$^{c\text{-}src}$ in neuronal growth cones and processes, with the antigen particularly concentrated in growth cones of long neurites. pp60$^{c\text{-}src}$ in growth cone membranes was an active tyrosine-specific protein kinase with elevated tyrosine-specific protein kinase activity and reduced electrophoretic mobility characteristic of the form of pp60$^{c\text{-}src}$ in central nervous system neurons. pp60$^{c\text{-}src}$ was present at lower levels in subcellular fractions from mature rat brain but synaptosomal membranes were not enriched. Preferential localization of an active form of pp60$^{c\text{-}src}$ in nerve growth cone membranes and persistence of pp60$^{c\text{-}src}$ in mature neurons suggest that this tyrosine kinase is important in growth cone-mediated neurite extension and synaptic plasticity.**

The normal cellular c-*src* gene encodes a 60-kDa tyrosine-specific protein kinase (pp60$^{c\text{-}src}$) that is homologous to the transforming protein of Rous sarcoma virus (1–4). pp60$^{c\text{-}src}$ is highly conserved in evolution and exhibits homology with growth factor receptor/tyrosine kinases, leading to the suggestion that pp60$^{c\text{-}src}$ plays a crucial role in the growth or development of animal cells. However, despite intensive investigation, the biological role of pp60$^{c\text{-}src}$ remains unknown.

Analysis of pp60$^{c\text{-}src}$ expression in the developing nervous system has indicated that pp60$^{c\text{-}src}$ is probably important in neuronal differentiation. pp60$^{c\text{-}src}$ is expressed in a wide variety of cell types, but highest levels are present in embryonic nervous tissues (5–12). Two phases of pp60$^{c\text{-}src}$ expression in the neural lineage have been identified by immunocytochemical staining of developing embryos. An early phase of pp60$^{c\text{-}src}$ expression occurs in neuroectodermal cells of gastrulae coincident with commitment to the neural pathway (9). A later phase of pp60$^{c\text{-}src}$ expression occurs in terminally differentiating neuronal cells at about the time they cease dividing and extend processes (neurites) (10, 11). Similarly, c-*src* transcripts in *Drosophila* have been shown to accumulate at two stages: in early embryos and postmitotic neural tissues of pupae (12). A key role for pp60$^{c\text{-}src}$ in neuronal cells is underlined by the recent finding that certain neurons and neuroblastoma cells express a special form of pp60$^{c\text{-}src}$ with elevated protein kinase activity (7, 8, 13, 14). Alternative RNA splicing is responsible for the presence of six additional amino acids in the form of pp60$^{c\text{-}src}$ that shows high activity (15, 16); this form is preferentially expressed in the central nervous system (17). Restricted localization of pp60$^{c\text{-}src}$ to a functionally specialized region of the central nervous system neuron would provide major insight into its biological function. Yet, the complexity of cell types and processes in histological sections of the neural retina and cerebellum, in which pp60$^{c\text{-}src}$ expression has been characterized immunocytochemically, precluded conclusions about its subcellular location (10, 11).

Located at the tip of the outgrowing neurite is the nerve growth cone, a motile structure rich in cytoskeletal elements, that guides the neurite through the developing tissue. In this report we demonstrate that the activated form of pp60$^{c\text{-}src}$ is highly concentrated in membranes of a subcellular fraction enriched in nerve growth cones from fetal rat brain, and pp60$^{c\text{-}src}$ antibodies preferentially bind the growth cone and proximal axon shaft of neurons in culture. These results suggest that pp60$^{c\text{-}src}$ is important in growth cone-mediated axon extension, a process that depends on selective cell-surface adhesion and cytoskeletal modulation.

MATERIAL AND METHODS

Subcellular Fractionation of Rat Brain and Preparation of Membranes. Brains of day-18 rat fetuses (E18) were fractionated by the procedure of Pfenninger *et al.* (18). Adult rat cortex was fractionated essentially as described by Cohen *et al.* (19). Membranes from subcellular fractions were prepared as follows and collected by centrifugation at 100,000 × *g* for 30 min after each step: hypotonic lysis in 6 mM Tris, pH 8.3/0.5 mM EDTA; salt extraction in 20 mM Hepes, pH 7.4/0.3 M Na_2SO_4 to remove adsorbed protein; washing in 20 mM Tris, pH 7.4/10 mM 2-mercaptoethanol/1 mM $MgCl_2$/1 mM EGTA/pepstatin A at 1 μg/ml; and resuspension in 20 mM Tris, pH 7.4/1 mM $MgCl_2$/pepstatin A at 1 μg/ml. Buffers were supplemented with 10 mM NaF, 200 μM Na_3VO_4, aprotinin at 100 kallikrein inhibitor units/ml, and 0.005% (wt/vol) leupeptin.

Immunoblotting. Proteins were electrophoresed under reducing conditions on 8.5% $NaDodSO_4$/polyacrylamide gels (7 cm) and transferred electrophoretically to nitrocellulose. Blots were incubated with anti-*src* mouse monoclonal antibody 327 (from J. Brugge, State University of New York at Stony Brook) and the Fab fragment of ^{125}I-labeled goat anti-mouse IgG (14). The *src* antibody was raised against bacterially expressed v-*src*-encoded protein (20). Bands were scanned using an LKB Ultrascan XL laser densitometer. Band intensities were linearly related to antigen concentration and were within the linear response of the film (21). Protein was measured by the method of Lowry *et al.* (22). Phospholipid phosphorus was measured as described by Ames and Dubin (23).

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Abbreviation: pp60$^{c\text{-}src}$, c-*src*-encoded 60-kDa tyrosine-specific protein kinase.

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Preparation of Cell Extracts and Immune Complex Protein Kinase Assays. The mouse C1300 neuroblastoma cell line Neuro-2a was obtained from the American Type Culture Collection. The NIH (pMcsrc/COS)A cell line, which overexpresses $pp60^{c\text{-}src}$ from a cloned c-*src* gene, was obtained from D. Shalloway (Pennsylvania State University, University Park, PA). Cell lines were propagated in culture (14). Extracts (1–2 mg/ml) were prepared from cultured cells, and membrane fractions were prepared by solubilization in 10 mM potassium phosphate buffer, pH 7.2/1% (wt/vol) Nonidet P-40/0.5% (wt/vol) sodium deoxycholate/0.15 M KCl/1 mM EDTA/7.5 mM EGTA/10 mM NaF/200 μM Na_3VO_4/1 mM ATP/aprotinin at 100 kallikrein inhibitor units per ml. Extracts were clarified by centrifugation at 16,000 × *g* for 15 min at 4°C. $pp60^{c\text{-}src}$ was immunoprecipitated from extracts in antibody excess with *src* monoclonal antibody 327 and goat anti-mouse IgG–agarose (14). Half the immunoprecipitate was used to measure $pp60^{c\text{-}src}$ kinase activity, and the other half was used to quantitate $pp60^{c\text{-}src}$ protein by immunoblotting. $pp60^{c\text{-}src}$ kinase activity was measured in the immune complexes under initial velocity conditions by using the synthetic peptide human angiotensin I (20 mM; Sigma) as phosphoacceptor, adenosine 5′-[γ-^{32}P]triphosphate (46 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) and manganese chloride (5 mM) (14).

Indirect Immunofluorescence Staining of Retinal Cell Cultures. Minced neural retinas from day-6 chick embryos were grown on glass precoated with poly(DL-ornithine) and laminin (GIBCO) for 18 hr in Ham's F-12 medium supplemented with transferrin at 100 μg/ml, Na selenite at 5 ng/ml, and insulin at 5 μg/ml (24). Cells were fixed in 4% (wt/vol) formaldehyde/0.01% (wt/vol) glutaraldehyde for 50 min at room temperature and washed in 0.1 M glycine hydrochloride in phosphate-buffered saline and then in 2% (vol/vol) sheep serum. Freeze-thaw permeabilization was done by immersing the cells in liquid nitrogen and thawing them at room temperature. Cells were incubated with a polyclonal rabbit antibody raised against bacterially expressed v-*src* protein (25) for 2 hr at room temperature (IgG, 80 μg/ml). After being washed in phosphate-buffered saline, cells were incubated with rhodamine-conjugated goat anti-rabbit IgG (Miles) for 1 hr, washed, mounted in glycerol, and scored under epifluorescence illumination. For antigen blocking, 25 μg of IgG was preincubated with 20 μg of purified $pp60^{v\text{-}src}$ antigen in 50 μl for 2 hr at room temperature, and then IgG was diluted to 80 μg/ml with phosphate-buffered saline before application to the cells. A monoclonal antibody directed against the 200-kDa subunit of neurofilament protein was from J. Wood (Sandoz Institute for Medical Research, London).

RESULTS

$pp60^{c\text{-}src}$ Is Enriched in a Growth Cone Membrane Fraction. $pp60^{c\text{-}src}$ was measured in subcellular brain fractions isolated from 18-day rat embryos (18). The fractionation steps included the following: (*i*) tissue homogenization, (*ii*) centrifugation (1660 × *g*) to produce a low-speed pellet and supernatant, and (*iii*) fractionation of the supernatant on a three-step discontinuous-sucrose density gradient, which at the 0.32 M/0.75 M-sucrose interface yielded the A fraction, the fraction at least 70% enriched in nerve growth cones (18). Because $pp60^{c\text{-}src}$ is known to be associated with the plasma membrane (26–28), membranes were prepared from each fraction, and equal amounts of membrane protein were analyzed for $pp60^{c\text{-}src}$ concentration by immunoblotting with *src* monoclonal antibodies (20). Membranes from the A fractions contained the highest levels of $pp60^{c\text{-}src}$ (Fig. 1). The $pp60^{c\text{-}src}$ content of A-fraction membranes was ≈9-fold greater than homogenate membranes (Table 1). This enrichment factor varied between 7- and 13-fold among four different

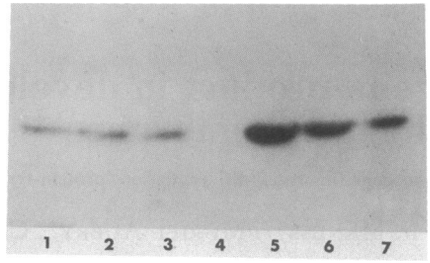

FIG. 1. Immunoblot analysis of $pp60^{c\text{-}src}$ in subcellular fractions from fetal rat brain (E18). Membrane proteins (100 μg) from each fraction and the soluble fraction from the sucrose gradient were electrophoresed and subjected to immunoblotting analysis with *src* monoclonal antibody 327. Lanes: 1, homogenate; 2, low-speed supernatant; 3, low-speed pellet; 4, soluble fraction from the gradient (0.32 M sucrose); 5, growth cone A fraction (0.32 M/0.75 M-sucrose interface); 6, B fraction (0.75 M/1 M-sucrose interface); and 7, C fraction (1 M/2.66 M-sucrose interface). Autoradiographic exposure time was 17 hr.

preparations. The enrichment of $pp60^{c\text{-}src}$ in A-fraction membranes was ≈50-fold when compared with total homogenate protein. Little enrichment in $pp60^{c\text{-}src}$ was seen in the membrane fraction of the low-speed pellet, which contained unlysed cells and cell bodies (Fig. 1). The relatively large fraction of total $pp60^{c\text{-}src}$ protein in this fraction may be due to contamination with neurite and growth cone elements resulting from incomplete tissue homogenization. More extensive homogenization caused lysis of growth cone particles, causing their redistribution into gradient fractions B and C. $pp60^{c\text{-}src}$ was not detected in the soluble fraction from the gradient.

Approximately 32% of the total $pp60^{c\text{-}src}$ protein recovered from the sucrose gradient partitioned into A-fraction membranes (Table 1). Membranes isolated from fractions B and C contained 20% and 48% of total $pp60^{c\text{-}src}$ protein, respectively, but $pp60^{c\text{-}src}$ was not as highly concentrated in these membranes as in A-fraction membranes. Fraction-B membranes are derived from small neuronal and glial fragments and Golgi cisternae, whereas fraction C membranes are derived from neurite shafts, rough endoplasmic reticulum, and mitochondria (18, 29). To evaluate the extent to which the presence of $pp60^{c\text{-}src}$ in B- and C-fraction membranes reflected contamination by lysed growth cone particles, the immunoblots were reprobed with mouse monoclonal antibody 5B4. This antibody recognizes a growth-associated glycoprotein (185–255 kDa) that has been shown by immunofluorescence to be concentrated in the nerve growth cone and the contiguous shaft of the axon and that is enriched in A-fraction membranes (30).

Like $pp60^{c\text{-}src}$, the 5B4 antigen exhibited its highest enrichment in membranes from the growth cone A fraction (Table 1). A lower, but significant enrichment of 5B4 antigen in membranes from the B fraction indicated the presence of growth cone membranes. Little 5B4 antigen was seen in membranes from the C fraction, indicating that $pp60^{c\text{-}src}$ in C-fraction membranes is probably not derived from growth cones.

The enrichment of $pp60^{c\text{-}src}$ in A-fraction membranes did not appear to be due to greater amounts of contaminating nonmembrane protein in the membranes from fractions B and C, shown by comparing levels of $pp60^{c\text{-}src}$ with respect to phospholipid phosphorus (Table 1). The ratio of $pp60^{c\text{-}src}$ to phospholipid was greatest in A-fraction membranes, consistent with the interpretation that $pp60^{c\text{-}src}$ is present at an elevated concentration in growth cone membranes. However, enrichment of $pp60^{c\text{-}src}$ per phospholipid content in A-fraction membranes was lower than when expressed relative to protein content. This difference could be due in part

Table 1. Relative levels of pp60$^{c\text{-}src}$ in subcellular membrane fractions from fetal and adult rat brain

	pp60$^{c\text{-}src}$ per mg of protein	Total mg of protein	Total pp60$^{c\text{-}src}$	pp60$^{c\text{-}src}$ per phospholipid P_i	5B4 antigen per mg of protein
Fetal rat brain (E18)					
Homogenate	1.0	333	333	1.0	1.0
Low-speed pellet	2.3	88	202	2.8	2.2
Low-speed supernatant	1.5	122	183	2.1	2.1
Sucrose density gradient					
A fraction (0.32 M/1 M-interface)	9.0	4.3	39	5.1	4.7
B fraction (0.75 M/1 M-interface)	3.7	6.5	24	3.6	2.5
C fraction (1 M/2.66 M-interface)	2.8	20.4	57	3.1	0.4
Adult rat brain					
Homogenate	1.0	82	82	1.0	—
Low-speed pellet	1.5	30	45	1.3	—
Low-speed supernatant	0.8	22	17	0.7	—
Sucrose density gradient					
0.32 M/1.0 M-interface	0.9	11	10	0.8	—
1.0 M/1.2 M-interface (synaptosomes)	1.5	2	3	1.3	—

pp60$^{c\text{-}src}$ was measured in membranes (100 μg) by scanning densitometrically the 60-kDa bands on the autoradiogram shown in Fig. 1 and one similar to Fig. 2*A*. 5B4 antigen in fetal brain fractions was measured by reprobing the same immunoblot with monoclonal antibody 5B4. Relative levels of pp60$^{c\text{-}src}$ and 5B4 antigen were normalized to values measured in homogenate membranes. Total protein represents values for preparations from 250 fetal and 2 adult rat brains.

to the high phospholipid-to-protein content of the growth cone membranes (18, 30).

pp60$^{c\text{-}src}$ Is Present at Lower Levels in Synaptosomal Membranes. To analyze the expression of pp60$^{c\text{-}src}$ in brain after maturation of synapses, pp60$^{c\text{-}src}$ levels were measured in subcellular fractions isolated from adult rat brain (19). The fractionation steps included the following: (*i*) tissue homogenization, (*ii*) low-speed centrifugation (1400 × *g*) to produce a low-speed pellet and supernatant, and (*iii*) fractionation of the supernatant on a two-step discontinuous-sucrose density gradient yielding a fraction at the 1 M/1.2 M-sucrose interface that is enriched in pinched-off synaptic terminals (synaptosomes). The enrichment of synaptosomes in the 1 M/1.2 M-interface fraction was confirmed by electron microscopy. Equal amounts of membrane protein from each fraction were subjected to immunoblotting with the *src* monoclonal antibody (Fig. 2*A*). Little enrichment of pp60$^{c\text{-}src}$ per protein relative to homogenate membranes was found in synaptosomal membranes (Table 1). The enrichment factor ranged from 1- to 2-fold among four preparations. Total pp60$^{c\text{-}src}$ protein in the synaptosomal membrane fraction was substantially lower than in membranes from the 0.32 M/1 M-sucrose interface fraction, which contained heterogeneous membranes and myelin (19). However, pp60$^{c\text{-}src}$ was not enriched in either fraction. High levels of total pp60$^{c\text{-}src}$ protein were observed in membranes from the low-speed pellet. This may reflect either incomplete cell lysis or localization in membranes of cell bodies. Detectable levels of pp60$^{c\text{-}src}$ were not found in the soluble fraction from the gradient.

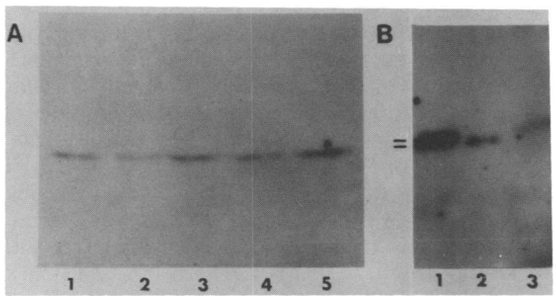

FIG. 2. Immunoblot analysis of pp60$^{c\text{-}src}$ in subcellular fractions from adult rat brain. (*A*) Membrane proteins (100 μg) from adult brain fractions were electrophoresed on 7-cm gels and subjected to immunoblotting analysis with *src* monoclonal antibody 327. Lanes: 1, homogenate; 2, low-speed supernatant; 3, low-speed pellet; 4, 0.32 M/1 M-sucrose interface containing heterogenous membranes and myelin; 5, synaptosome fraction (1/1.2 M-sucrose interface). Autoradiographic exposure time was 17 hr. (*B*) Membrane proteins were electrophoresed on 18-cm gels and subjected to immunoblotting analysis with *src* monoclonal antibody 327. Lanes: 1, growth cone A fraction (100 μg); 2, NIH (pMcsrc/COS)A fibroblast extract (20 μg); and 3, synaptosomal fraction (100 μg). Autoradiographic exposure time was 16 hr.

The amounts of pp60$^{c\text{-}src}$ per protein content in selected fractions of fetal (E18) and adult brain membranes were compared with each other by immunoblotting. pp60$^{c\text{-}src}$ was measured by densitometric scanning of the autoradiographic bands and was expressed in arbitrary units of absorbance per mg of protein. pp60$^{c\text{-}src}$ in homogenate membranes (1.0 unit of pp60$^{c\text{-}src}$ per mg) and low-speed supernatant membranes (2.3 units per mg) from fetal rat brain (E18) was significantly greater than in homogenate membranes (0.4 unit/mg) and low-speed supernatant membranes (0.3 unit/mg) from adult brain. pp60$^{c\text{-}src}$ in the growth cone-enriched A-fraction membranes (9.0 units/mg) was 15-fold greater than in synaptosomal membranes (0.6 unit/mg). This ratio of pp60$^{c\text{-}src}$ in growth cone membranes compared with synaptosomal membranes ranged from 9- to 19-fold among four different preparations. In preliminary experiments, the enrichment of pp60$^{c\text{-}src}$ in A-fraction membranes was found to be ≈7-fold greater than in membranes from a low-speed supernatant of neural tubes dissected from day-11 rat embryos. At this stage, the neural tube contains a higher proportion of proliferating progenitors than sprouting or mature neurons.

No change in the distribution of pp60$^{c\text{-}src}$ was seen when fetal and adult rat brain tissues were fractionated without sodium orthovanadate, an inhibitor of phosphotyrosine phosphatases (31). Electron microscopy of the A fraction and synaptosomal fraction prepared in the presence and absence of sodium orthovanadate confirmed that the fractionation behavior of growth cone particles or synaptosomes was not obviously affected.

Anti-pp60$^{c\text{-}src}$ Antibodies Preferentially Bind to Growth Cones. To determine whether the enrichment of pp60$^{c\text{-}src}$ in the A fraction reflected a preferential disposition within growth cones, pp60$^{c\text{-}src}$ was localized in primary cultures of chick retinal neurons by indirect immunofluorescence using a polyclonal antibody specific for pp60$^{c\text{-}src}$ in chicken cells

(25). By 18 hr after plating, cultures of retinal explants from day-6 chick embryos contained numerous cells with a neuronal structure. Cells with neurites greater than two cell bodies in length stained positively with a monoclonal antibody directed against neurofilament protein. Approximately half of the cells (47%) had neurites greater than 10 cell bodies in length and were probably retinal ganglion neurons (32). $pp60^{c\text{-}src}$-specific immunofluorescence was seen in growth cones, neurites, and cell bodies; however, cells with longer neurites showed more intense staining in the growth cone and contiguous region of the neurite than in the region of the neuritic shaft more distal to the growth cone (Fig. 3*a*). This pattern was seen in 70% of cells (142/204) with neurites greater than 10 cell bodies in length, in 49% of cells (74/152) with neurites 5–10 cell bodies in length, and in 22% of cells (26/119) with neurites 2–5 cell bodies in length. Neuronal cells that did not exhibit a growth cone enrichment in $pp60^{c\text{-}src}$ antigen appeared uniformly fluorescent in the growth cone and neuritic shaft. Staining was effectively blocked by preincubating the antibody with purified antigen, except for low-level residual fluorescence in the cell body (Fig. 3*c*). No staining was seen with preimmune serum.

$pp60^{c\text{-}src}$ in Growth Cone Membranes Is a Tyrosine Kinase of High Specific Activity. To determine whether $pp60^{c\text{-}src}$ in growth cone and synaptosomal membranes represented the form with elevated protein kinase activity characteristic of certain neuronal cells (7, 8, 13), the specific activity of $pp60^{c\text{-}src}$ in these fractions was determined in an immune complex protein kinase assay and compared with that of the activated form of $pp60^{c\text{-}src}$ expressed in the mouse C1300 neuroblastoma cell line Neuro-2a. We showed previously that $pp60^{c\text{-}src}$ in this neuroblastoma cell line exhibits elevated specific activity, due to an increased V_{max} of the enzyme (14). When equal amounts of $pp60^{c\text{-}src}$ protein were compared, activities from growth cone membranes (392 fmol of P_i per min), synaptosomal membranes (419 fmol per min), and neuroblastoma cells (403 fmol per min) were equivalent. Lower activity was seen with an equal amount of $pp60^{c\text{-}src}$ from NIH (pMcsrc/COS)A fibroblasts (135 fmol per min). The neuronal form of $pp60^{c\text{-}src}$ from brain is the product of an alternatively spliced messenger RNA, which directs the insertion of six amino acids into the NH_2-terminal domain of the protein (15, 16), resulting in a slightly reduced electrophoretic mobility of the protein (7, 16). The electrophoretic mobilities of $pp60^{c\text{-}src}$ in growth cone and synaptosomal membrane fractions were retarded relative to $pp60^{c\text{-}src}$ in fibroblasts (Fig. 2*B*). The $pp60^{c\text{-}src}$ band from the growth cone A fraction was broad, indicating the additional presence of either the lower molecular weight form or a modified form of neuronal $pp60^{c\text{-}src}$. These results demonstrate that $pp60^{c\text{-}src}$ in the growth cone membrane is an activated form of tyrosine-protein kinase that is likely to result from alternative splicing. The specific activity of $pp60^{c\text{-}src}$ does not appear to change with maturation of nerve terminals.

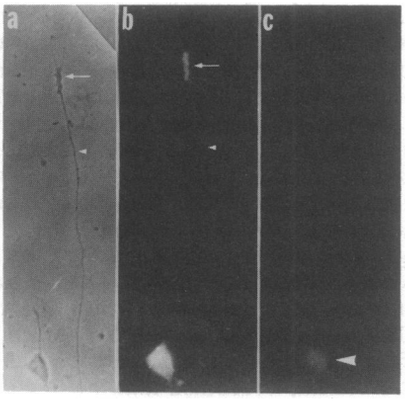

FIG. 3. Indirect immunofluorescence staining of retinal neurons with anti-$pp60^{c\text{-}src}$ antibodies. (*a*) Phase contrast. (*b*) Growth cone, contiguous region of neurite and cell body labeled with anti-$pp60^{c\text{-}src}$ antibodies. (*c*) Competition control photographed under conditions identical to *b*, showing a low level of staining only in the cell body when the primary antibody was preincubated with purified antigen. Arrow, growth cone; small arrowhead, neurite; large arrowhead, cell body. (×860.)

DISCUSSION

We showed that $pp60^{c\text{-}src}$ is enriched in the membrane of rat embryonic nerve growth cones, where it is a tyrosine-protein kinase of high specific activity. $pp60^{c\text{-}src}$ persists at lower levels in adult rat brain but does not appear to be concentrated in synaptic terminals. These results strongly indicate a subcellular site for $pp60^{c\text{-}src}$ action and suggest that $pp60^{c\text{-}src}$ plays an important biological role in nerve growth cones.

These observations are consistent with previous immunocytochemical staining results showing $pp60^{c\text{-}src}$ in process-rich regions of the developing neural retina, cerebellum, and telencephalon (10, 11) and, in addition, demonstrate that $pp60^{c\text{-}src}$ is enriched in growth cone membranes. $pp60^{c\text{-}src}$ in the subcellular growth cone fraction was a highly active tyrosine kinase, suggesting that it phosphorylates substrates implicated in the specialized functions of the growth cone. $pp60^{c\text{-}src}$ appeared less abundant in membranes of the axonal shaft, as indicated by a lower level of immunofluorescence staining in regions of the neurite distal to the growth cone and by its lower enrichment in membranes from the fetal rat brain C fraction, which contains neurite fragments. $pp60^{c\text{-}src}$ in the axon shaft may subserve a biological function or represent protein that remains in the axolemma after the growth cone has migrated onward during axon extension. The presence of the activated form of $pp60^{c\text{-}src}$ kinase in adult rat brain indicates that $pp60^{c\text{-}src}$ also functions within the mature neuron. The finding that $pp60^{c\text{-}src}$ was not enriched in synaptosomal membranes suggests that this function of $pp60^{c\text{-}src}$ in the adult central nervous system may not lie in synaptic transmission. Instead, $pp60^{c\text{-}src}$ in adult brain may be involved in neuronal plasticity, and its precise function could be similar to that required, to a much greater degree, in growth cone-mediated neurite extension.

Our previous studies on $pp60^{c\text{-}src}$ in cultured dorsal root ganglion neurons showed immunofluorescence staining in growth cones, neurites, and cell bodies without an enrichment in growth cones (33). It was recently shown that $pp60^{c\text{-}src}$ in the peripheral nervous system is primarily in the form of slightly lower molecular weight than the major form in the central nervous system (17). Thus, only the higher molecular weight form could be enriched in growth cones. We also note that detergent was used to permeabilize the dorsal root ganglion cells, conditions that could have partially solubilized $pp60^{c\text{-}src}$ from the growth cone membrane despite mild aldehyde fixation. Retinal explant cells were permeabilized by freeze-thaw, a procedure less likely to cause antigen loss.

The nerve growth cone is guided toward its synaptic target by dynamic changes in cell adhesion and cytoskeletal structure. We speculate that $pp60^{c\text{-}src}$ in the growth cone may modulate cell–substratum adhesion or motility, important features of growth cone migration necessary for neurite outgrowth. It is noteworthy that the activated form of $pp60^{c\text{-}src}$ is expressed concurrently with the extension of processes during neuronal differentiation of an embryonal carcinoma cell line (34). Moreover, the highly homologous retroviral transforming protein $pp60^{v\text{-}src}$ induces neurite outgrowth in the rat pheochromocytoma cell line PC12, demonstrating that a *src* tyrosine kinase can elicit outgrowth of neuronal processes (35). A role for $pp60^{v\text{-}src}$ in cell adhesion is supported by its

localization in adhesion plaques of transformed fibroblasts (36–38) and its ability to cause loss of zonula adherens junctions between epithelial cells (39). Deregulation of normal cell growth by the transforming protein $pp60^{v\text{-}src}$ may involve a related mechanism. Disruption of cell–cell or cell–substratum contact by $pp60^{v\text{-}src}$ may lead to anchorage-independence of cell growth, the parameter of transformation that correlates best with tumorigenicity (40). $pp60^{c\text{-}src}$ in nonneuronal cells including platelets (41), erythrocytes (42), fibroblasts (1–3), astrocytes (7), and presumptive neuroectodermal cells of gastrulating embryos (9) may have analogous roles in cell adhesion, maintenance of cell shape, and morphogenetic movements that share components with growth cone-mediated axon extension.

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