MOLECULAR AND CELLULAR BIOLOGY, Nov. 1987, p. 4115–4117
0270-7306/87/114115-03$02.00/0

Vol. 7, No. 11

NOTES

An Altered Form of pp60$^{c\text{-}src}$ Is Expressed Primarily in the Central Nervous System

JEAN M. LE BEAU,* OTMAR D. WIESTLER, AND GERNOT WALTER

Department of Pathology, University of California, San Diego, La Jolla, California 92093

Received 4 June 1987/Accepted 5 August 1987

The expression of two forms of pp60$^{c\text{-}src}$, pp60 and pp60^{+}, was measured in the central nervous system (CNS) and the peripheral nervous system. Both forms were expressed in the CNS, whereas only pp60 was primarily detected in the peripheral nervous system. Our findings suggest that pp60^{+} may play a role in events important to the CNS.

pp60$^{c\text{-}src}$, the cellular homolog of the transforming protein of the Rous sarcoma virus, pp60$^{v\text{-}src}$, is a phosphoprotein with a high tyrosine kinase activity in the brain (3, 5, 9, 11). Immunocytochemical studies have shown the appearance of pp60$^{c\text{-}src}$ in postmitotic neurons at the onset of differentiation, and this expression is maintained in fully differentiated, mature, and functional neurons (4, 11). Similarily, biochemical analyses of pp60$^{c\text{-}src}$ in the central nervous system (CNS) have demonstrated the expression of this protein coincident with the occurrence of neuronal differentiation (3, 4, 12; O. Wiestler and G. Walter, unpublished data). These findings implicate the expression of pp60$^{c\text{-}src}$ in CNS neuronal differentiation and function.

The pp60$^{c\text{-}src}$ protein is expressed as two structurally distinct forms in CNS neurons. One form, designated here as pp60, has the same electrophoretic mobility on sodium dodecyl sulfate-polyacrylamide gels as does pp60$^{c\text{-}src}$ from fibroblasts or astrocytes (2). The other form, designated pp60^{+}, has a slightly higher molecular weight and an altered pattern of phosphorylation in the N-terminal half of the molecule and to date has only been observed in CNS neurons and neuroblastoma cells (1, 2). Although very similar in size, pp60 and pp60^{+} can be separated and quantitated by polyacrylamide gel electrophoresis (Wiestler and Walter, unpublished data).

In this study, we used in vitro kinase assays and Western blotting (immunoblotting) techniques to determine whether both forms of pp60$^{c\text{-}src}$ are also expressed in the peripheral nervous system (PNS). Five adult Sprague-Dawley rats (Charles River Breeding Laboratories, Inc.) weighing 180 to 200 g were anesthetized by intraperitoneal injection of Nembutal (50 mg/kg of body weight). CNS (forebrain, midbrain, cerebellum, hippocampus, brain stem, and spinal cord), PNS (cervical sympathetic ganglion, adrenal medulla, and dorsal root ganglia pooled from the cervical, thoracic, and lumbar spinal regions), and nonneural tissues (liver, adrenal cortex, and muscle) were carefully dissected free from each animal, pooled, and homogenized with a Dounce homogenizer in 0.5 to 1.0 ml of RIPA lysis buffer (10 mM sodium phosphate [pH 7.2], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM dithiothreitol, 50 μM leupeptin). Homogenates were cleared by centrifugation at 10,000 × *g* for 30 min at 2°C. Protein concentrations were determined with a Lowry assay kit (Sigma Chemical Co.). For in vitro kinase assays, tissue lysates (100 μg of total protein) were incubated with 1.0 μl of ascites fluid of monoclonal antibody GD11, followed by the addition of Formalin-fixed *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring). GD11 is a monoclonal antibody which recognizes an epitope near the amino terminus of the pp60$^{c\text{-}src}$ molecule (8). The samples were then washed and incubated for 30 min at 30°C with 25 μl of kinase assay mixture containing 10 mM phosphate buffer (pH 6.6), 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM leupeptin, and 10 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham Corp.). The kinase reaction was stopped by the addition of 50 μl of electrophoresis sample buffer, and the samples were boiled for 5 min and loaded onto a sodium dodecyl sulfate-15% polyacrylamide gel. To achieve good separation of the two forms of pp60$^{c\text{-}src}$, we ran the gels at a constant current of 30 mA for 6 h, which is 3 h longer than required for the dye front to reach the end of the gel.

Figure 1A shows the reaction products detected by autoradiography. Two distinct forms of the autophosphorylated protein, pp60 and pp60^{+}, were identified in immunoprecipitates from extracts of forebrain, hippocampus, cerebellum, midbrain, brain stem, and spinal cord (lanes a through f). The upper, slower-migrating form, pp60^{+}, which may correspond to the neuron-specific c-*src* protein described by Brugge and co-workers (2), was generally absent from PNS lysates, although it was present in CNS lysates (lanes g through i). Only one PNS sample, cervical ganglion (lane h), showed a trace amount of kinase activity in the pp60^{+} region. Autophosphorylated pp60$^{c\text{-}src}$ was not detectable in the adrenal cortex, liver, and muscle (data not shown). These findings were similar to those obtained with mouse tissues (J. Le Beau and G. Walter, unpublished data).

For Western blots, tissue lysates were diluted in an equal volume of 2× electrophoresis sample buffer, boiled for 5 min, and electrophoresed on sodium dodecyl sulfate-15% polyacrylamide gels as described above. The proteins were then transferred onto nitrocellulose paper for 3.5 h at 225 mA in a solution containing 25 mM Tris (pH 8.3), 200 mM glycine, and 20% methanol. Nonspecific protein-binding sites were saturated by overnight incubation of the nitrocel-

* Corresponding author.

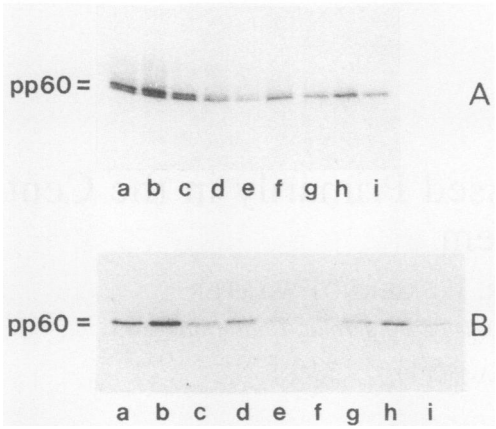

FIG. 1. Expression of pp60$^{c\text{-}src}$ in adult rat CNS and PNS tissues. (A) Analysis of pp60$^{c\text{-}src}$ kinase activity. In vitro kinase assays were carried out with tissue extracts and analyzed by polyacrylamide gel electrophoresis as described in the text. (B) Western blot analysis of nervous system tissue samples. Total extract protein (25 to 35 μg) was electrophoresed on sodium dodecyl sulfate-15% polyacrylamide gels, transferred onto nitrocellulose paper, and analyzed for pp60 and pp60$^+$ protein. Lanes (A and B): a, forebrain; b, hippocampus; c, cerebellum; d, midbrain; e, brain stem; f, spinal cord; g, dorsal root ganglia; h, cervical ganglia; i, adrenal medulla.

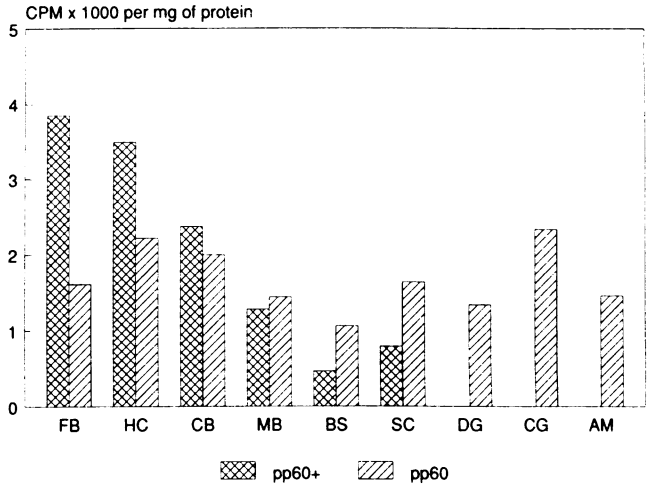

FIG. 2. Quantification of pp60$^{c\text{-}src}$ kinase activity in adult rat CNS and PNS tissues. Bands corresponding to pp60 and pp60$^+$ were excised from 15% polyacrylamide gels, and the ^{32}P content was determined in a scintillation counter. The kinase activity is expressed as counts per minute per milligram of total extract protein. FB, Forebrain; HC, hippocampus; CB, cerebellum; MB, midbrain; BS, brain stem; SC, spinal cord; DG, dorsal root ganglia; CG, cervical ganglia; AM, adrenal medulla.

lulose membrane in a mixture containing 10 mM Tris (pH 7.5), 170 mM NaCl, and 3% Carnation nonfat milk. For detection of pp60$^{c\text{-}src}$, the filters were incubated with a 1:250 dilution of monoclonal antibody 327 ascites fluid (7) for 2 h at room temperature and washed extensively in a mixture containing 150 mM KCl, 10 mM imidazole (pH 7.3), 5 mM $MgCl_2$, 0.3% Tween 20, and 0.1% $CaCl_2$. Since monoclonal antibody 327 has a low affinity to protein A, a 1:500 dilution of goat anti-mouse immunoglobulin G (Cooper Biomedical) was added as a bridging antibody for 2 h at room temperature. After being washed extensively, the membranes were reacted with 10 μCi of ^{125}I-labeled protein A (Amersham) for 2 h at room temperature, washed extensively, and exposed with a Kodak XAR-5 autoradiography film.

The detection of pp60$^+$ and pp60 on immunoblots of crude CNS and PNS extract protein is shown in Fig. 1B. We observed pp60 and pp60$^+$ in all CNS tissue extracts examined. A longer exposure of the film was required for the detection of both forms of pp60$^{c\text{-}src}$ in the spinal cord and brain stem (data not shown). pp60 was also detected in all PNS tissues examined; however, cervical ganglion was the only sample which showed a small amount of protein in the pp60$^+$ region (lane h). Because the pp60$^{c\text{-}src}$ signal is lower in PNS tissues than in CNS tissues, we can not exclude the possibility of a small amount of pp60$^+$ being present in dorsal root ganglia and adrenal medulla. The regional difference in the presence or absence of pp60$^+$ and pp60 in the nervous system corresponds to the observed kinase activity of the two forms of *src* protein. This finding suggests that the general absence of pp60$^+$ from the PNS appears to be determined at the level of protein synthesis.

To determine the amount of kinase activity in CNS and PNS tissue extracts, we excised bands corresponding to pp60 and pp60$^+$ from the gel and quantitated them by scintillation spectrometry. The forebrain and hippocampus were enriched in pp60$^+$ kinase activity compared with pp60 activity (Fig. 2). In contrast, the cerebellum and midbrain showed comparable levels of kinase activity in the two forms of *src* protein, whereas the brain stem and spinal cord predominantly contained pp60. Although the PNS samples lacked pp60$^+$ kinase activity, the pp60 activity was generally comparable with that observed in CNS tissues. In fact, cervical ganglion showed the highest amount of pp60 activity of all tissues examined. The phosphorylation of enolase, an exogenous substrate for pp60$^{c\text{-}src}$, was similar to the autophosphorylation of pp60 and pp60$^+$ (data not shown).

Our findings suggest that the CNS-specific form of the *src* protein, pp60$^+$, may not be necessary for mature PNS functioning but rather may be important for events that are unique to CNS neurons. For example, the PNS and CNS differ in their ontogeny in that the CNS is derived from the neural tube and the PNS is derived from the neural crest (6). Therefore, the differential expression of the two forms of *src* in the PNS and CNS may reflect different developmental requirements of these two distinctly derived neural tissues. Neurons in the CNS are also capable of elaborate demonstrations of plasticity. The CNS-specific form of c-*src* may be important in events that are associated with neuronal plasticity. In contrast to pp60$^+$, pp60 is expressed at comparable levels in both the CNS and PNS, suggesting that pp60 may be important in events common to both nervous systems, such as nerve conduction.

We thank both Sarah Parsons and Joan Brugge for generously providing us with monoclonal antibodies GD11 and 327, respectively.

This work was supported by American Cancer Society grant CD-276 to G.W., by postdoctoral fellowships from the Deutsche Forschungsgemeinschaft to O.D.W., and by Public Health Service grant NS07078-10 from the National Institute of Neurological and Communicative Disorders and Stroke to J.M.L.

LITERATURE CITED

1. **Bolen, J. B., N. Rosen, and M. A. Israel.** 1985. Increased pp60$^{c\text{-}src}$ tyrosyl kinase activity in human neuroblastomas is associated with amino-terminal tyrosine phosphorylation of the

src gene product. Proc. Natl. Acad. Sci. USA **82**:7275–7279.
2. **Brugge, J. S., P. C. Cotton, A. E. Queral, J. N. Barrett, D. Nonner, and R. W. Keane.** 1985. Neurones express high levels of a structurally modified activated form of pp60$^{c\text{-}src}$. Nature (London) **316**:554–557.
3. **Cotton, P. C., and J. S. Brugge.** 1983. Neural tissues express high levels of the cellular *src* gene product pp60$^{c\text{-}src}$. Mol. Cell. Biol. **3**:1157–1162.
4. **Fults, D. W., A. C. Towle, J. M. Lauder, and P. F. Maness.** 1985. pp60$^{c\text{-}src}$ in the developing cerebellum. Mol. Cell. Biol. **5**:27–32.
5. **Jacobs, C., and H. Ruebsamen.** 1983. Expression of pp60$^{c\text{-}src}$ protein kinase in adult and fetal human tissue: high activities in some sarcomas and mammalian carcinomas. Cancer Res. **43**:1696–1702.
6. **Jacobsen, M.** 1978. Developmental neurobiology, p. 5–20. Plenum Press, New York.
7. **Lipsich, L. A., A. J. Lewis, and J. S. Brugge.** 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma viruses. J. Virol. **48**:352–360.
8. **Parsons, S. J., D. J. McCarley, V. W. Raymond, and J. T. Parsons.** 1986. Localization of conserved and nonconserved epitopes within the Rous sarcoma virus-encoded *src* protein. J. Virol. **59**:755–758.
9. **Schartel, M., and A. Barnekow.** 1982. The expression in eukaryotes of a tyrosine kinase which is reactive with pp60$^{c\text{-}src}$ antibodies. Differentiation **23**:109–114.
10. **Sorge, J. P., L. K. Sorge, and P. F. Maness.** 1985. pp60$^{c\text{-}src}$ is expressed in human fetal and adult brain. Am. J. Pathol. **119**: 151–157.
11. **Sorge, L. K., B. T. Levy, and P. F. Maness.** 1984. pp60$^{c\text{-}src}$ is developmentally regulated in the neural retina. Cell **36**:249–257.
12. **Vardimon, L., L. E. Fox, and A. A. Moscona.** 1986. Accumulation of c-*src* mRNA is developmentally regulated in embryonic neural retina. Mol. Cell. Biol. **6**:4109–4111.