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Developmental Expression of Two Forms of pp60$^{c\text{-}src}$ in Mouse Brain

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The expression during mouse development of two forms of protein-tyrosine kinase pp60$^{c\text{-}src}$, pp60 and pp60^{+}, was investigated. At embryonic day 9 (E9), only pp60 was detected in whole-brain lysates. A trace of pp60^{+} was first seen at E10. In E18 embryos and in adults, pp60^{+} was the predominant form of pp60$^{c\text{-}src}$ in forebrain and midbrain lysates.

It has been reported that the brains of mammals, birds, and fish contain more pp60$^{c\text{-}src}$, the protein-tyrosine kinase encoded by the proto-oncogene c-*src*, than most other organs and tissues (4, 5, 8, 14, 16), indicating that pp60$^{c\text{-}src}$ may serve a special function in the brain. Neurons contain an additional form of pp60$^{c\text{-}src}$ which is structurally distinct from the form found in astrocytes and fibroblasts. The difference involves an altered pattern of phosphorylation (1, 2) and the insertion of six amino acids in the *N*-terminal half of the molecule (12). Interestingly, this neuronal form is expressed only in central nervous system neurons and very little, if at all, is detected in the peripheral nervous system (7).

The expression of c-*src* in neural tissue is developmentally regulated. In developing chick neural retina, pp60$^{c\text{-}src}$ and mRNA first appear on embryonic day 3.5 (E3.5) and persist through adulthood (15, 18). In chicken brain, c-*src* kinase activity is very low on E3 and E5, increases strongly between E6 and E9, and decreases slightly before hatching (4). High levels of pp60$^{c\text{-}src}$ have been found in cultures prepared from embryonic-chicken dorsal root ganglia (10) and in the neural ectoderm of early chicken embryos during gastrulation (11). These studies did not distinguish between the two forms of pp60$^{c\text{-}src}$ with respect to their developmental regulation. Therefore it was not clear which form, if any, predominated during development.

We report here on the first comprehensive investigation of the expression in vivo of the two forms of pp60$^{c\text{-}src}$, here designated pp60 and pp60^{+}, during development. We quantitatively determined the amounts and kinase activities of these proteins in different parts of the developing mouse brain. Mouse embryos (C57BL/6) were removed at E9 to E18. For E9, E10, and E11 embryos, the head region, consisting mainly of brain tissue, was separated from the spinal column and the remainder of the embryo. At E12, forebrain and midbrain were dissected out and assayed separately, and at E14 and later stages, cerebellum was analyzed in addition to forebrain and midbrain. Lysates from forebrain, midbrain, cerebellum, and liver (which served as a control) were prepared in RIPA buffer (17). Immunoprecipitations with monoclonal antibody GD11 (13), in vitro kinase assays (autophosphorylation and phosphorylation of enolase), and immunoblotting experiments were carried out as previously described (7). A low kinase activity of pp60 was detected in lysates of heads of E9 embryos (Fig. 1, lane a). The central nervous system-specific pp60^{+} kinase activity was clearly seen in E11 heads. However, a longer exposure showed a trace amount of activity at E10. A strong increase in kinase activity, in particular of pp60^{+}, occurred between E12 (lanes d and e) and E14 (lanes f and g). The kinase activity in spinal cord (lanes i to m) resembled that in head and brain with respect to kinase levels and to the appearance of pp60^{+} at E10. E12 liver contained only a low activity of pp60 and no pp60^{+} activity (lane n). Total embryos at E8.5 and the internal organs of E9 to E11 fetuses also contained low amounts of pp60 and no pp60^{+} (data not shown). The activity of enolase phosphorylation generally paralleled the autophosphorylation activity.

The relative contributions of pp60 and pp60^{+} autophosphorylation activities were assayed in immunoprecipitates from extracts of forebrain, midbrain, and cerebellum of animals between E14 and postnatal day 28 (P28). A peak of pp60^{+} activity was reached at E18 in forebrain and midbrain (Fig. 2). The decline in pp60^{+} activity after E18 could be caused by an increasing number of glial cells, which do not contain pp60^{+}. In contrast to forebrain and midbrain, cerebellum contained approximately equal amounts of pp60 and pp60^{+} between E14 and P28. Note that the values in Fig. 2 for E9 to E11 were determined with extracts from the entire heads, as shown in Fig. 1.

To characterize the structural relationship between pp60 and pp60^{+}, organotypic cultures were prepared from P7 cerebella (17) and labeled with ^{32}P. pp60 and pp60^{+} were

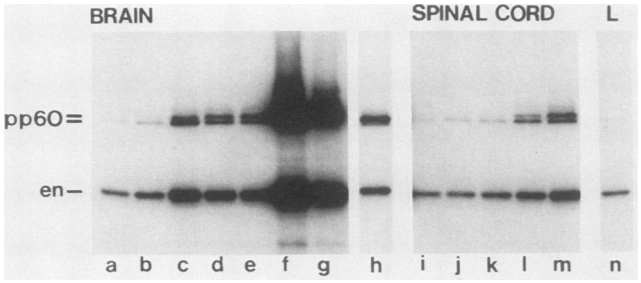

FIG. 1. Induction of pp60$^{c\text{-}src}$ kinase in early embryonic central nervous system. Kinase assays were carried out with central nervous system extracts from E9 to E14 mouse embryos and analyzed by polyacrylamide gel electrophoresis. Lanes: a, E9 head; b, E10 head; c, E11 head; d, E12 midbrain; e, E12 forebrain; f, E14 midbrain; g, E14 forebrain; h, shorter exposure of lane f sample; i, E9 spinal column; j, E10 spinal column, tail section containing no spinal cord; k, E10 spinal column, proximal segment with spinal cord; l, E11 spinal column; m, E12 spinal cord; n, E12 liver (L). en, Enolase.

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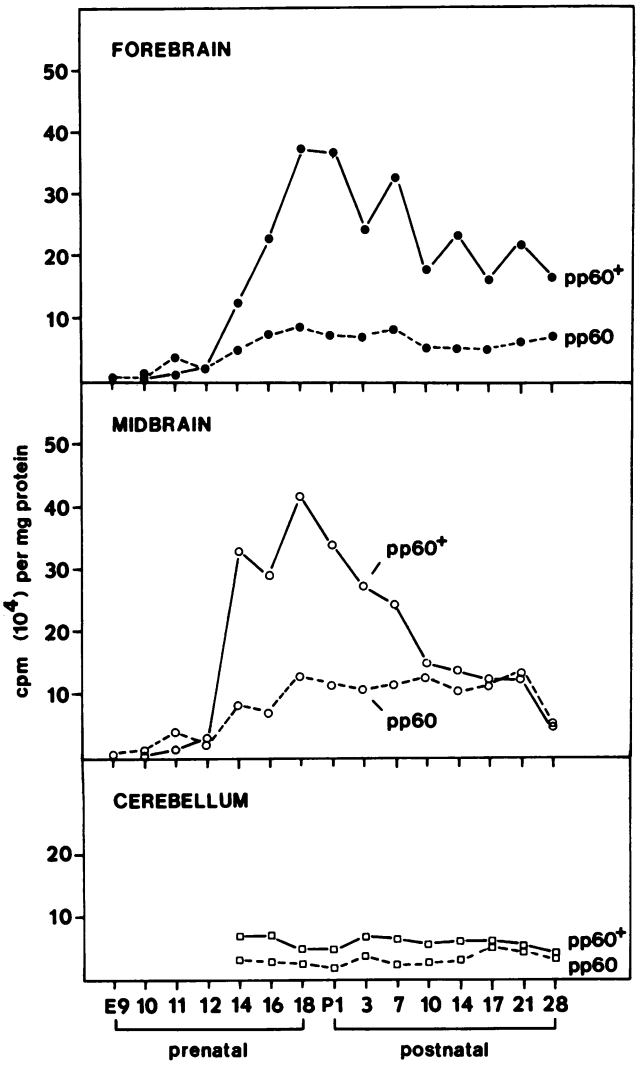

FIG. 2. Tyrosine kinase activities of the two forms of pp60$^{c\text{-}src}$ during mouse brain development. Neuronal Src protein (pp60$^+$) and its counterpart (pp60) were excised from 15% polyacrylamide gels and counted in a scintillation counter. Kinase activity is expressed as counts per minute per milligram of extract protein. The data for early stages E9, E10, and E11 are derived from head samples which contained the whole brain.

immunoprecipitated and analyzed by one-dimensional V8 protease mapping (3). Both proteins were cleaved into four fragments, designated V1 to V4. The V2 fragments were identical in size, whereas V1, V3, and V4 from pp60 migrated faster than those from pp60$^+$ (data not shown). Similar V8 digests of two forms of pp60$^{c\text{-}src}$ from rat brain were reported by Brugge et al. (2).

The amounts of pp60 and pp60$^+$ proteins in the developing brain were determined by immunoblotting. Brain and liver samples from representative prenatal and postnatal stages are shown in Fig. 3. We found small amounts of pp60 and pp60$^+$ in E12 forebrain and midbrain (lanes b and f); protein levels increased 10- to 15-fold between E12 and P1 (lanes d and g) and were maximal during the perinatal period (P1), with pp60$^+$ predominant in both tissues (lanes d and g). A postnatal decline was seen in forebrain and midbrain (lanes e and h). Cerebellum contained similar quantities of pp60 and pp60$^+$ in P3 and P21 samples. Immunoblots did not detect pp60 in P1 and P21 liver (lanes k and l), indicating that the concentration in hepatocytes is at least 1 order of magnitude lower than that in adult brain. Stage- and tissue-specific differences of pp60$^{c\text{-}src}$ kinase activity could reflect either differences in the steady-state levels of pp60$^+$ and pp60 proteins or differences in the specific activity of the two forms. By quantification of the experimental data (from densitometry and measurement of radioactivity of excised gel bands), we found that the observed developmental changes in kinase activity were paralleled by similar changes in protein amounts (data not shown). Therefore, the developmental changes of pp60 and pp60$^+$ kinase activities appeared to occur, at least in part, at the level of protein synthesis. We noticed that the ratio of pp60$^+$ to pp60 was higher in immunoblots, carried out with monoclonal antibody 327 (9), than in kinase reactions, carried out with monoclonal antibody GD11. The reason for this difference is unclear.

Between E10 and E18, telencephalon, mesencephalon, and metencephalon give rise to forebrain, midbrain, and cerebellum, respectively. This development involves active proliferation of neuroblasts in the ventricular layer, migration of young neurons out of this layer, and differentiation into germinal, mantle, and marginal layers. On E18, the brain is essentially complete in its general topography (6). Thus, while pp60$^+$ activity rose sharply between E12 and E18, concomitant increases in proliferation, differentiation, and migration were taking place in all parts of the brain. Therefore, we cannot attribute the increase in pp60$^+$ to any of these activities. Previous studies have indicated that the Src protein is expressed only at a postmitotic stage during neuronal development (15). Our data are compatible with this idea where pp60$^+$ is concerned, since an increasing number of cells in forebrain, midbrain, and cerebellum enter the postmitotic state between E12 and E18. However, it seems unlikely that pp60 is expressed only in postmitotic cells, since it was already expressed at E8.5 in the neural tube. We are presently trying to identify by in situ hybridization and immunohistochemistry which cells are responsible for the increase in pp60 and pp60$^+$ during development.

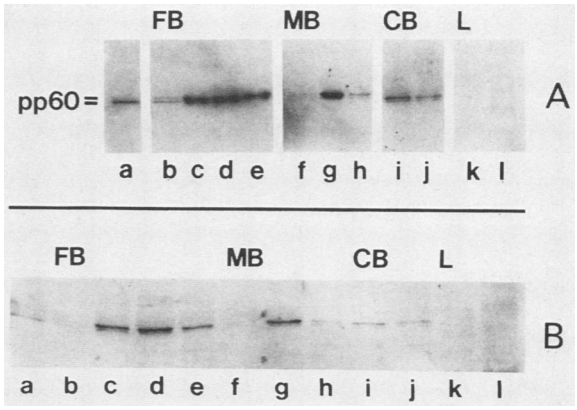

FIG. 3. Western blot (immunoblot) analysis of representative tissue samples. Total protein (10 μg) was electrophoresed on sodium dodecyl sulfate-15% polyacrylamide gels, transferred onto nitrocellulose paper, and analyzed for pp60$^+$ and pp60 proteins with monoclonal antibody 327 (9). (A and B) Separate blots from the same samples. Lanes: a, E11 head; b, E12 forebrain (FB); c, E16 forebrain; d, P1 forebrain; e, P21 forebrain; f, E12 midbrain (MB); g, P1 midbrain; h, P21 midbrain; i, P3 cerebellum (CB); j, P21 cerebellum; k, P1 liver (L); l, P21 liver.

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