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Localization and Phosphorylation of Abl-Interactor Proteins, Abi-1 and Abi-2, in the Developing Nervous System

Kevin D. Courtney,* Matthew Grove,* Hendrika Vandongen,*,† Antonius Vandongen,*,† Anthony-Samuel LaMantia,‡ and Ann Marie Pendergast*,[1]

**Department of Pharmacology and Cancer Biology and †Department of Neurobiology, Duke University Medical Center, Durham, North Carolina 27710; and ‡Department of Cell and Molecular Physiology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599*

Abl-interactor (Abi) proteins are targets of Abl-family nonreceptor tyrosine kinases and are required for Rac-dependent cytoskeletal reorganization in response to growth factor stimulation. We asked if the expression, phosphorylation, and cellular localization of Abi-1 and Abi-2 supports a role for these proteins in Abl signaling in the developing and adult mouse nervous system. In mid- to late-gestation embryos, *abi-2* message is elevated in the central and peripheral nervous systems (CNS and PNS). *Abi-1* mRNA is present, but not enhanced, in the CNS, and is not observed in PNS structures. Abi proteins from brain lysates undergo changes in apparent molecular weight and phosphorylation with increasing age. In the postnatal brain, *abi-1* and *abi-2* are expressed most prominently in cortical layers populated by projection neurons. In cultured neurons, Abi-1 and Abi-2 are concentrated in puncta throughout the cell body and processes. Both Abi and Abl proteins are present in synaptosomes and growth cone particles. Therefore, the Abi adaptors exhibit proper expression patterns and subcellular localization to participate in Abl kinase signaling in the nervous system.

INTRODUCTION

The Abl-interactor proteins, Abi-1 (e3B1) and Abi-2, are common downstream targets of the Abl-family of nonreceptor tyrosine kinases (NRTKs), which includes c-Abl and Arg (Dai and Pendergast, 1995; Shi *et al.*, 1995; Wang *et al.*, 1996a; Biesova *et al.*, 1997). Abi proteins bind to and are substrates of c-Abl and Arg tyrosine kinases and are implicated in the regulation of cell growth and transformation (Dai and Pendergast, 1995; Shi *et al.*, 1995; Wang *et al.*, 1996a; Dai *et al.*, 1998). Abi-1 has also been linked to cytoskeletal reorganization through its interactions with Sos-1 and Eps8, a substrate of several receptor tyrosine kinases, including epidermal growth factor receptor (EGFR) (Scita *et al.*, 1999). A complex of Abi-1, Sos-1, and Eps8 exhibits activity as a guanine nucleotide exchange factor for the Rac GTPase that regulates membrane ruffling and lamellipodia formation (Scita *et al.*, 1999). Microinjection of fibroblasts with anti-Abi-1 antibodies resulted in abrogation of Rac-dependent membrane ruffling in response to platelet derived growth factor (PDGF) stimulation (Scita *et al.*, 1999). Abi proteins are therefore linked to both receptor- and nonreceptor tyrosine kinase- as well as GTPase-mediated signalling events.

Abi-1 and Abi-2 share significant identity, exhibiting greater than 90% conservation in their amino-termini and in their carboxy-terminal SH3 domains. Multiple isoforms of both proteins have been identified, resulting from alternative splicing events (Biesova *et al.*, 1997; Taki *et al.*, 1998). Interactions with c-Abl and Arg tyrosine kinases are mediated through the SH3 domain and proline-rich regions of Abi-1 and Abi-2 (Dai and Pendergast, 1995; Shi *et al.*, 1995; Wang *et al.*, 1996a). Abi-1 and Abi-2 also contain a homeobox–homology region (HHR) that retains several critical residues of the helix–turn–helix DNA-binding motif common to home-

[1] To whom correspondence and reprint requests should be addressed. Fax: (919) 681-7148. E-mail: pende014@mc.duke.edu.

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odomains (Dai and Pendergast, 1995; Shi *et al.,* 1995). *Abi* genes are widely expressed in mice and humans, with highest mRNA levels observed in the brain (Dai and Pendergast, 1995; Shi *et al.,* 1995). *Abi-2* is the mammalian ortholog of the *Xenopus laevis xlan4* gene. *xlan4* is developmentally regulated, with increased transcript levels at the neurula stage localizing to dorsal axial structures, which are principally comprised of the developing CNS (Reddy *et al.,* 1992). In larvae and adults, *xlan4* is primarily expressed in the brain (Reddy *et al.,* 1992). *abi-2,* as well as the related *abi-1,* may therefore play a role in neuronal development and function.

Increasing evidence points to roles for Abl- and Src-family NRTKs in neuronal development and axonogenesis. Src-family kinases (including c-Src, Fyn, Lyn, and c-Yes) have been implicated in neuronal development, differentiation, and neurite outgrowth (Grant *et al.,* 1992; Beggs *et al.,* 1994; Ignelzi *et al.,* 1994). Src has been shown to regulate *N*-methyl-d-aspartate (NMDA) receptor activity, and Lyn and Fyn have been linked to alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor signaling and regulation (Yu *et al.,* 1997; Hayashi *et al.,* 1999; Narisawa-Saito *et al.,* 1999). Expression of either c-Abl or Arg is essential for proper neurulation in mice (Koleske *et al.,* 1998). Genetic studies of *Drosophila abl* (*D-abl*) have revealed roles for *D-abl* in axonogenesis and growth cone pathfinding (Hu and Reichardt, 1999; Van Etten, 1999).

Little is known regarding potential common targets of the c-Abl, Arg, and Src tyrosine kinases in neuronal development. Abi proteins are functionally linked to Abl and Src-family tyrosine kinases. Abi-1 and Abi-2 have been shown to regulate the transforming capacity of Abl proteins, and expression of oncogenic forms of Abl and Src tyrosine kinases downregulates Abi protein levels (Dai and Pendergast, 1995; Shi *et al.,* 1995; Dai *et al.,* 1998). Abi proteins may function as targets for c-Abl and Arg tyrosine kinases in neuronal cells. To this end, we examined and compared the expression patterns of *abi-1* and *abi-2* in the developing nervous system, and the subcellular localization of Abi- and Abl-family proteins in neurons. Our results show that *abi-1* and *abi-2* exhibit unique expression patterns in early CNS and PNS development, but similar localization in the postnatal brain. Moreover, Abi adaptor proteins are expressed with c-Abl and Arg in the neuron cell body, at synapses, and in growth cone particles, where they may function to transduce signaling events downstream of the Abl kinases or may contribute to the regulation of c-Abl and Arg kinase activity.

RESULTS

Abi Protein Expression during Embryogenesis and Postnatal Brain Development

We first asked whether Abi-1, Abi-2, and c-Abl proteins are present in developing and adult tissues, including the CNS. Lysates of mouse embryos of gestational age 10–16 days (E10–E16) were prepared and analyzed by immunoprecipitation and Western blotting techniques. Peak c-Abl expression is observed at the earliest ages examined (E10–E13), consistent with previous reports of *c-abl* transcript levels (Müller *et al.,* 1982) (Fig. 1A). Using anti-Abi serum 5421, which recognizes the protein products of both *abi-1* and *abi-2* (Dai *et al.,* 1998), multiple Abi protein bands are recognized from E10–E16 (Fig. 1A). c-Abl and Abi proteins are more highly expressed in lysates prepared from embryo heads than trunks. Among postnatal tissues examined, Abi protein is most highly expressed in the brain (data not shown). In late embryonic and postnatal brain lysates, Abi proteins undergo a shift in apparent molecular weight to faster migrating forms on reducing gels with increasing age of mice (Fig. 1B). c-Abl expression diminishes in postnatal brain lysates in older mice (Fig. 1B).

The multiple bands and changing apparent molecular weight observed for Abi proteins in Western blots of mouse embryo and postnatal brain lysates may reflect differences in expression of Abi-1 and Abi-2, multiple alternative splice variants of both Abi-1 and Abi-2, or changes in posttranslational modifications of these proteins. To determine whether there are differences in the expression of Abi-1 and Abi-2 at different ages, we generated antibodies which specifically recognize Abi-1 or Abi-2 by immunoprecipitation (M. Grove, R. C. Quackenbush, and A. M. Pendergast, unpublished observations). Both Abi-1 and Abi-2 are expressed in embryos and in post-natal brains, with Abi-1- and Abi-2-specific antibodies recognizing multiple bands corresponding to Abi-1 or Abi-2, respectively (Figs. 1C and 1D). Both Abi proteins undergo a marked shift in mobility over time in postnatal brain lysates. The multiple bands observed in Western blots and immunoprecipitation experiments are specific for the Abi proteins, as confirmed by loss of Abi-2 protein bands in lysates prepared from brains of *abi-2*$-/-$ mice, with retention of Abi-1 protein expression (M. Grove and A. M. Pendergast, unpublished data).

Changes in phosphorylation of Abi proteins could contribute to the observed shifts in protein mobility. In this regard, Abi-1 has been shown to become hyper-

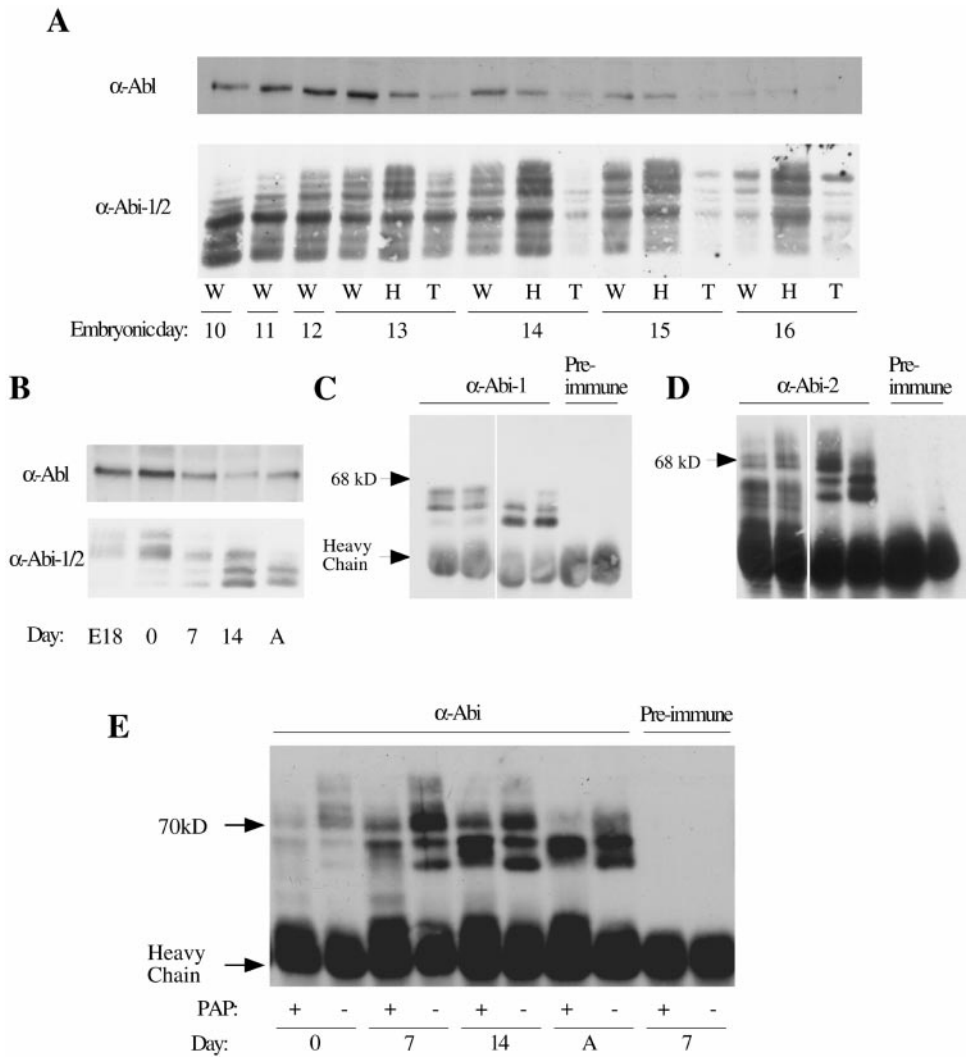

FIG. 1. Abi and c-Abl proteins are expressed during embryogenesis, and Abi proteins undergo changes in phosphorylation and apparent molecular weight in the developing postnatal brain. (A, B) c-Abl and Abi proteins are detected in E10-E16 mouse embryos and in E18 and postnatal brain lysates. Lysates (30 μg of tissue per lane) were electrophoresed on SDS–PAGE gels, transferred to nitrocellulose membranes, and blotted with anti-Abi serum 5421 or a monoclonal antibody recognizing c-Abl. (A) c-Abl expression decreases in embryos late in gestation (top). Abi proteins are expressed from E10–E16, with higher protein levels observed in head (H) fractions than in embryo trunks (T) (bottom). W, whole embryo lysate. (B) c-Abl levels appear higher in late embryonic (E18) and neonatal (0, 7 days) brain lysates than in brains from older mice (14 days; A, adult) (top). Abi proteins undergo an apparent molecular weight shift in the developing postnatal brain (bottom). (C, D) Lysates (2 mg total protein) of embryos (E12, E14) or postnatal brains (7 days; A, adult) were immunoprecipitated with antibodies recognizing Abi proteins or with normal rabbit serum (Pre-immune). (C) Immunoprecipitation of embryo and brain lysates with anti-Abi-1-specific rabbit polyclonal antibodies or with preimmune serum reveals a shift in the mobility of Abi-1. (D) Immunoprecipitation of embryo and brain lysates with anti-Abi-2-specific polyclonal antibody reveals that Abi-2 is shifted to lower molecular weight forms between P7 and adult mice. (E) PAP treatment of Abi proteins immunoprecipitated from postnatal brain lysates leads to changes in protein migration on reducing gels, consistent with dephosphorylation of Abi proteins in neonatal brain lysates.

phosphorylated on serine following mitogenic stimulation of serum-starved fibroblasts that overexpress EGFR (Biesova *et al.*, 1997). To test whether phosphorylation of Abi proteins contributes to the observed changes in apparent molecular weight, immunoprecipitated Abi-1 and Abi-2 proteins from brain lysates were treated with potato acid phosphatase (PAP) or calf intestinal alkaline phosphatase (CIP). PAP (Fig. 1E) or CIP (data not shown) treatment elicits a marked shift in the electrophoretic migration of the highest molecular

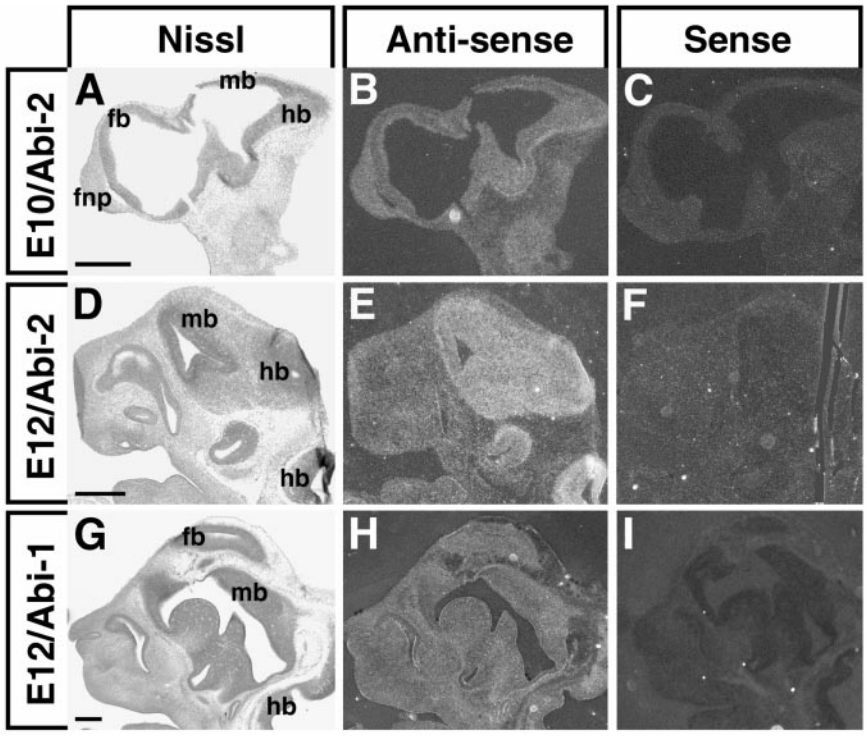

FIG. 2. *abi-2* expression in mouse embryos at E10 and E12 is concentrated in the CNS. *In situ* hybridization was carried out with sense and antisense probes to *abi-1* and *abi-2* on sagittal sections of E10 and E12 mouse embryo heads. (A–C) *abi-2* message at E10 is concentrated in the frontonasal prominence (fnp) and the neuroepithelium of the forebrain (fb), midbrain (mb), and hindbrain (hb) of the E10 mouse embryo. *abi-2* hybridization by (B) antisense and (C) sense probes is presented. (D–F) *abi-2* message is prominent in the CNS at E12. (E) Antisense and (F) sense riboprobes were used. (G–I) *abi-1* expression is uniform throughout sagittal sections of E12 mouse embryo heads. Hybridization by (H) antisense and (I) sense probes for *abi-1* is presented. (A, D, G) Bright field images are shown. Scale bars, 1 mm.

weight forms of Abi proteins in P0 and P7 brain lysates, as well as changes in the mobility of Abi proteins in lysates from older mice. PAP treatment of postnatal brain lysates reveals changes in Abi-2 phosphorylation in lysates from neonates compared to brains of older mice, while changes in Abi-1 phosphorylation are not observed by this method (data not shown). Because c-Abl levels diminish with age in post-natal brain lysates, we hypothesized that c-Abl-mediated tyrosine phosphorylation might contribute to the observed differences in Abi phosphorylation at different ages. However, we do not detect tyrosine phosphorylation of Abi proteins by Western blot of anti-Abi immunoprecipitates from postnatal brain lysates with anti-phosphotyrosine antibody 4G10 (data not shown).

Expression of abi-1 and abi-2 during Early Differentiation of the Nervous System

To more precisely localize Abi-1 and Abi-2 expression during development, we prepared frozen sections of mouse embryos and postnatal brains. Abi-1 and Abi-2 proteins could not be detected by immunohistochemistry using available Abi-1 and Abi-2 antibodies. We therefore performed *in situ* hybridization with sense and antisense probes specific for *abi-1* and *abi-2* transcripts.

We first examined *abi-1* and *abi-2* expression prior to the onset of cortical neurogenesis. At E10 the neuroepithelium of the presumptive forebrain is undergoing symmetrical cell division resulting in a population of pluripotent progenitor cells (Zindy *et al.*, 1997). At E10, *abi-2* mRNA detected with an antisense riboprobe appears more highly expressed in the neuroepithelium of the developing forebrain, midbrain, and hindbrain regions of the CNS, as well as in the frontonasal prominence, than in the adjacent cephalic and trunk mesenchyme (Fig. 2B). This elevated expression can be best appreciated by comparing the antisense probe-labeled section with the section labeled with an *abi-2* sense riboprobe (Figs. 2B and 2C). Although there is a slight amount of detectable labeling in the sense probe-la-

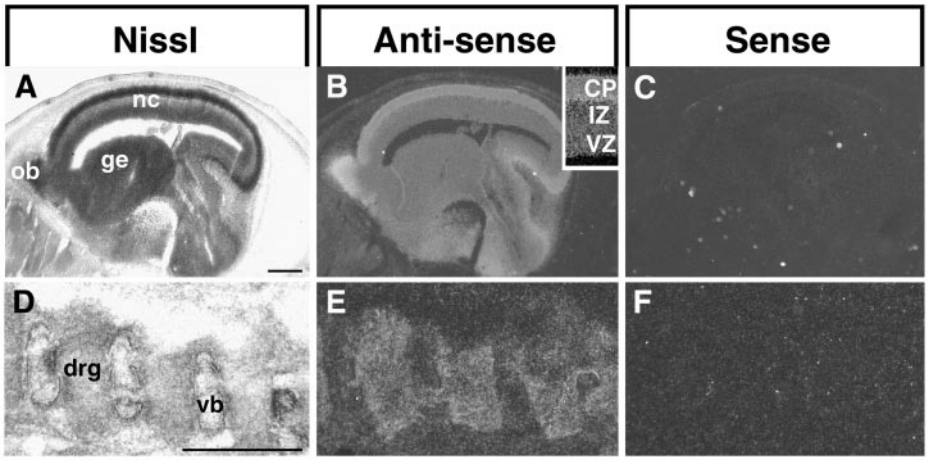

FIG. 3. *abi-2* message is enhanced in the CNS and PNS at E16. (A–C) Sagittal sections of E16 mouse embryo heads reveal prominent *abi-2* expression in the developing CNS. (B) *abi-2* levels as determined by hybridization with an antisense riboprobe appear higher in the cortical plate (CP) than in the underlying intermediate zone (IZ) or ventricular zone (VZ) (B, inset) and are elevated in the olfactory bulb (ob). ge, ganglionic eminence; nc, neocortex. (C) *abi-2* hybridization by a sense riboprobe is shown. (D–F) *abi-2* is prominently expressed in dorsal root ganglia (drg). vb, vertebral body. (E) Antisense and (F) sense riboprobes were used. (A, D) Bright field images are presented. Scale bars, 0.5 mm.

beled section, it is uniform throughout the embryo. Accordingly, *abi-2* expression is apparently enhanced in the developing brain at mid-gestation. *Abi-1* message is present in the developing CNS at E10, but does not appear more prominent in the neuroepithelium compared to the surrounding tissue (data not shown). Significantly, c-Abl and Arg are also prominently expressed in the neuroepithelium at E9–E10.25 (Koleske *et al.*, 1998).

By E12, following the onset of neurogenesis, *abi-2* message is enhanced throughout the developing CNS (Figs. 2D–2F). Elevated *abi-2* is detected in the developing brain and along the full length of the spinal cord (data not shown). *abi-1* does not appear to be enhanced in the CNS relative to other tissues at E12 (Figs. 2G–2I). At E16 *abi-2* hybridization remains enhanced throughout the CNS, but with apparent regional distinctions (Figs. 3A–3C). *Abi-2* message is particularly prominent in the olfactory bulb at this stage (Fig. 3B). Within the developing neocortex, *abi-2* mRNA is more highly expressed in the cortical plate than in the underlying intermediate zone (IZ) or ventricular zone (VZ) (Fig. 3B, inset). Enhanced expression of *abi-2* is not limited to the CNS. In the periphery, *abi-2* levels are also elevated in dorsal root ganglia (DRGs) at E16 (Figs. 3D–3F). At E16 hybridization to *abi-1* mRNA is detected throughout the CNS in an unrestricted fashion which is not elevated relative to surrounding tissues, similar to the expression pattern observed at E12 (data not shown). In contrast to *abi-2*, which is expressed in DRGs (Fig. 3E), *abi-1* is not detected in these PNS structures (data not shown).

abi-1 and abi-2 Expression in the Postnatal Brain

To determine whether *abi-1* and *abi-2* show prominent expression in specific regions of the postnatal brain, we performed *in situ* hybridization on brain sections from postnatal day 7 (P7) mice. At this stage of development, only specific neuronal populations continue to proliferate, including the cells of the dentate gyrus and the external granular layer of the cerebellum (Meller and Glees, 1969). While differences in expression between *abi-1* and *abi-2* are observed in the embryonic brain, *abi-1* and *abi-2* exhibit similar hybridization patterns in the P7 brain, with strongest expression in the cerebral cortex, hippocampus, dentate gyrus, olfactory bulb, and cerebellum (Figs. 4A–4F). The elevated expression of both genes in the cerebral cortex does not appear to be confined to particular layers. Similar expression patterns were observed for *abi-1* and *abi-2* in brain sections from P0 mice (data not shown).

The expression patterns of *abi-1* and *abi-2* in the brains of adult mice (>6 weeks) are similar to the patterns observed in the P7 brain. *Abi-2* transcripts are prominently expressed in the neocortex, hippocampus, and dentate gyrus (Figs. 5A and 5B). Again, *abi-2* message does not appear to be limited to particular layers of the neocortex (data not shown). In the cerebellum, *abi-2* appears highest in the Purkinje layer (Figs. 5E–5G). The

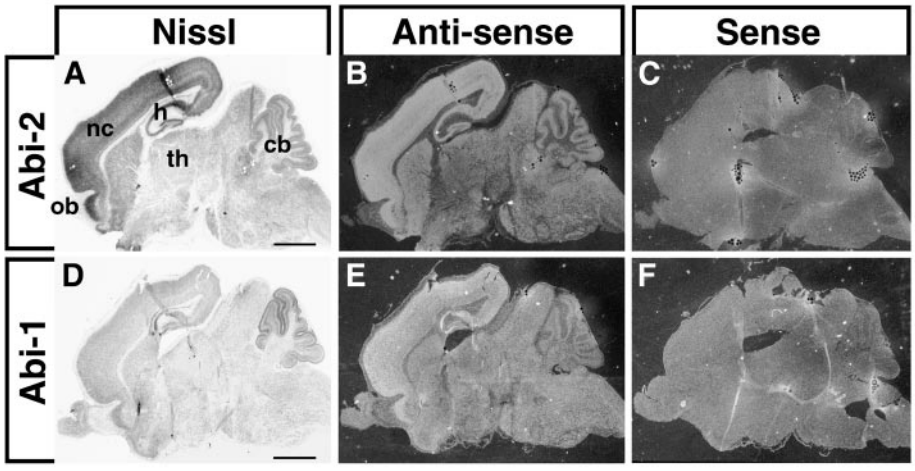

FIG. 4. *abi-1* and *abi-2* mRNAs are enhanced in specific regions of the mouse brain at postnatal day 7. Sagittal sections of brains from P7 mice were analyzed by *in situ* hybridization with probes against *abi-1* and *abi-2* mRNAs. (A–C) *abi-2* is elevated in the neocortex (nc), hippocampus (h), and cerebellum (cb). th, thalamus. (D–F) *abi-1* hybridization is also prominent in the neocortex, hippocampus, olfactory bulb, and cerebellum. Sections were hybridized with (B, E) antisense and (C, F) sense riboprobes. (A, D) Bright field images are shown. Scale bars, 2 mm.

mitral cell layer of the olfactory bulb also shows prominent *abi-2* hybridization (Figs. 5K–5M). Similar to *abi-2*, *abi-1* transcripts are expressed in the hippocampus and dentate gyrus (Figs. 5C and 5D). *abi-1* mRNA is also detected in the neocortex (data not shown). In the cerebellum, both the Purkinje layer and the granular layer show *abi-1* hybridization above background (Figs. 5H–5J). Like *abi-2*, *abi-1* is also prominent in the mitral cell layer of the olfactory bulb (Figs. 5N–5P).

Subcellular Localization of Fluorescently Tagged Abi-1 and Abi-2 in Cultured Neurons

Having identified the regional distribution of *abi-1* and *abi-2* messages in the nervous system at different developmental stages, we wished to examine the subcellular localization of Abi-1 and Abi-2 proteins within neurons. To localize Abi-1 and Abi-2 proteins within living cells, we generated fusion proteins of isoforms of Abi-1 and Abi-2 with enhanced yellow fluorescent protein (EYFP) and expressed these constructs in neurons and glial cells cultured from embryonic day 18 (E18) rat hippocampal tissue.

We first examined Abi-1 · EYFP and Abi-2 · EYFP expression in neurons transfected within a few days of plating (3–6 days in culture) and imaged live neurons the day after transfection. Neurons in culture for 2–6 days have extended axons and are undergoing rapid dendritic outgrowth and the completion of neurite differentiation (Dotti *et al.*, 1988; Pennypacker *et al.*, 1991). Abi-1 · EYFP and Abi-2 · EYFP exhibit a punctate pattern of expression in neurons transfected at 5–6 days in culture (Figs. 6B and 6C). This pattern of fluorescence is most prominent in the cell body (Figs. 6B and 6C, insets), but also extends into neurites and is suggestive of vesicular structures. Neurons transfected at 5 days in culture with EYFP alone do not exhibit the punctate distribution of fluorescence associated with Abi expression (Fig. 6A).

To localize Abi-1 · EYFP and Abi-2 · EYFP in mature neurons, we transfected neurons after 14 days in culture. Neurons reach maturation, with differentiated axons and dendrites, by 7 days in culture (Dotti *et al.*, 1988). Two weeks after plating, the neurons in this *in vitro* culture system have established multiple synaptic contacts and have extended long, thin axons that can be distinguished from dendrites (Dotti *et al.*, 1988). Abi-1 · EYFP expression is again most prominent in the cell body, where it retains the vesicular pattern observed in younger neurons (Fig. 6E, inset). This punctate pattern extends into multiple dendrites, where Abi-1 · EYFP is observed within the length of the dendrite, as well as at discrete points that appear to be associated with dendritic spines (Figs. 6E and 6F). Abi-1 · EYFP fluorescence is enhanced in association with a subset of spines, suggesting Abi-1 may concentrate at specific synapses (Fig. 6F, arrows). Abi-1 · EYFP is also present in the axon (Fig. 6E). The punctate localization of Abi-1 · EYFP is distinct from that of EYFP alone, which is expressed diffusely in the cell body and neurites (Fig. 6D). Under

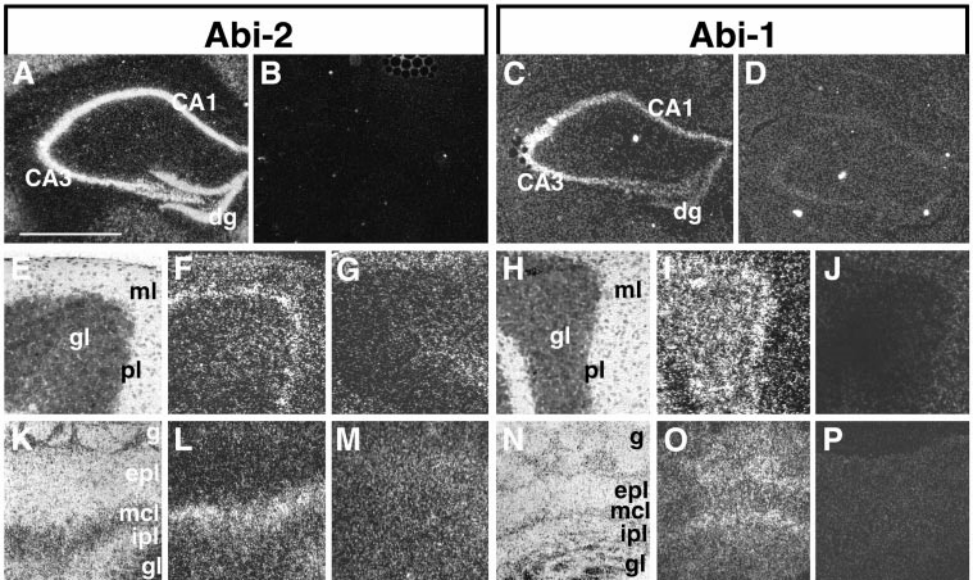

FIG. 5. *abi-1* and *abi-2* are concentrated in specific regions of the adult mouse brain. (A, B) Hybridization with (A) antisense and (B) sense probes to *abi-2* reveals expression in the CA1 and CA3 regions of the hippocampus and the dentate gyrus (dg) in >6-week-old mice. (C, D) Hybridization with (C) antisense and (D) sense probes to *abi-1* shows expression in the hippocampus and dentate gyrus. (E–G) (E) Bright-field and (F, G) dark-field images of a cerebellar folium are shown. (F) *abi-2* appears elevated in the Purkinje layer (pl) of the cerebellum by antisense hybridization. gl, granular layer; ml, molecular layer. (G) Hybridization with a sense control probe is shown. (H–J) *abi-1* appears concentrated in the Purkinje layer and the granular layer. (H) A bright field image is shown. (I, J) Dark-field images are provided for *abi-1* hybridization with (I) antisense and (J) sense probes. (K–M) *abi-2* appears concentrated in the mitral cell layer (mcl) of the olfactory bulb. epl, external plexiform layer; g, glomerulus; gl, granular layer; ipl, internal plexiform layer. (K) Bright-field and (L, M) dark-field images are shown. *abi-2* hybridization by (L) antisense and (M) sense probes is presented. (N–P) *abi-1* also appears elevated in the mitral cell layer of the olfactory bulb. (N) Bright-field and (O, P) dark-field images are provided. Scale bars, 0.5 mm.

similar culture and transfection conditions, we were unable to detect expression of Abi-2 · EYFP in neurons transfected after 2 weeks in culture.

Arg, the predominant Abl-family kinase in the brain, has previously been localized to the cytosol in neuroepithelial cells and transfected fibroblasts (Wang and Kruh, 1996; Koleske *et al.*, 1998). To examine whether Abi-1 · EYFP and Abi-2 · EYFP colocalize with the Arg kinase, transfected neurons were fixed and imaged by confocal microscopy following immunocytochemistry. Confocal microscopy revealed that endogenous Arg, Abi-1 · EYFP, and Abi-2 · EYFP are excluded from the nucleus in transfected neurons (data not shown). Endogenous Arg expression is observed throughout the cytosol in the cell body and extends into neurites. The punctate pattern of Abi-1 · EYFP and Abi-2 · EYFP expression colocalizes in part with Arg in the cell body and in neurites; however, overexpression of the Abi proteins does not sequester endogenous Arg protein into identically localized, discrete punctate structures (data not shown).

Subcellular Localization of Endogenous Abi-1, Abi-2, and Abl-Family Kinases in Fractionated Brain Lysates

We next localized endogenous Abi proteins within neuronal cell compartments. Because we were unable to localize Abi proteins within cultured neurons or tissue sections by indirect immunofluorescence using our anti-Abi antibodies, we examined endogenous Abi protein expression in lysates of fractions prepared from neonatal and adult rat brains by Western blotting with anti-Abi serum 5421. Fractionation of adult rat brain yielded the postnuclear supernatant and synaptosomes (synaptic terminals) (Strack *et al.*, 1997). Arg has previously been shown to be enriched in synaptosomes (Koleske *et al.*, 1998). Like Arg, the c-Abl tyrosine kinase and Abi adaptor proteins are expressed in synaptosomes (data not shown). To determine whether mammalian Abi and Abl-family proteins localize to growth cones, brains from neonatal (P3) rats were fractionated to yield growth cone particles and growth cone membranes (Patterson and Skene, 1999). A greater fraction of

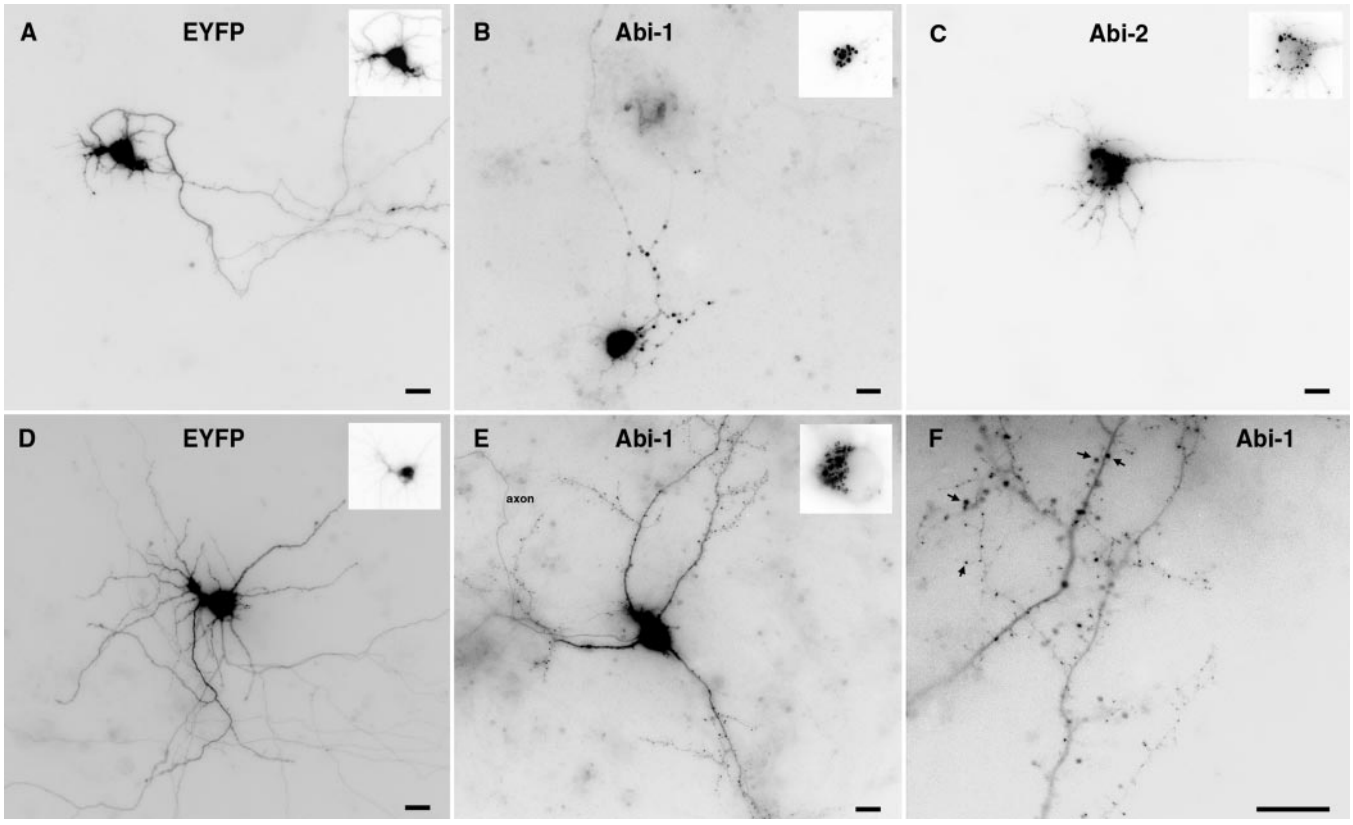

FIG. 6. Abi-1 · EYFP and Abi-2 · EYFP localize with a punctate distribution in the cell body and neurites of cultured neurons. (A–C) E18 rat hippocampal neurons cultured for 5–6 days were transfected with (A) EYFP, (B) Abi-1 · EYFP, or (C) Abi-2 · EYFP and imaged live by fluorescence microscopy. (A) EYFP is expressed throughout the neuron and appears uniformly distributed in the cell body (inset). (B) Abi-1 · EYFP exhibits a punctate distribution in the cell body (inset) and in neurites. (C) Abi-2 · EYFP expression also yields a punctate distribution in the cell body (inset) and in neurites. (D–F) Neurons cultured for 2 weeks were transfected with (D) EYFP or (E, F) Abi-1 · EYFP. (D, inset) EYFP is again expressed throughout the neuron. (E) Abi-1 · EYFP can be seen in dendrites and the axon (labeled). Abi-1 · EYFP is expressed in a punctate pattern in the cytosol (inset). Abi-1 · EYFP also localizes to structures that appear to be dendritic spines. (F) Magnification of (E) showing Abi-1 · EYFP expression in dendrites and apparent dendritic spines (arrows). Scale bars, 20 μm.

the Abi proteins is present in growth cone particles and growth cone membrane fractions compared to the supernatant (Fig. 7). Abl and Arg kinases are also present in these fractions, consistent with previous results which have localized *Drosophila* Abl to the growth cone (Henkemeyer *et al.,* 1987; Gertler *et al.,* 1989). Similarly, Src is enriched in growth cone preparations compared to the supernatant as previously reported (Bixby and Jhabvala, 1993). Thus, Abi proteins may be involved in NRTK signaling in the growth cones.

DISCUSSION

Mounting evidence implicates Abl- and Src-family NRTKs in nervous system development and neuronal function. Abi-family proteins have previously been identified as substrates and binding partners of c-Abl and Arg and as targets for degradation mediated by oncogenic forms of Abl and Src (Dai and Pendergast, 1995; Shi *et al.,* 1995; Dai *et al.,* 1998). Here we report the temporal and spatial distribution of *abi-1* and *abi-2* in the developing nervous system and the subcellular localization of Abi proteins in neurons. Our findings suggest that Abi-1 and Abi-2 may perform unique and shared functions in the nervous system that change during development. Changes in phosphorylation and apparent molecular weight of Abi proteins during CNS maturation suggest Abi-1 and Abi-2 may participate in developmentally regulated signaling events in neuronal cells.

Abi and c-Abl proteins are expressed in the CNS

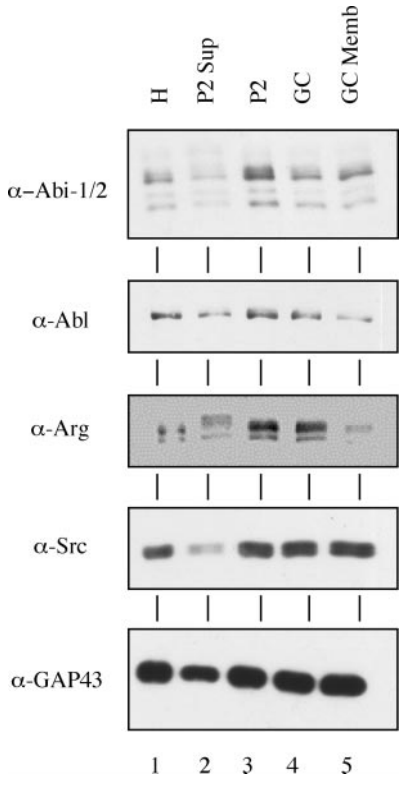

FIG. 7. Abi, c-Abl, and Arg proteins are expressed in growth cone particles. Lysates of postnatal day 3 rat forebrain subcellular fractions (10 μg total protein) were analyzed by Western blotting with antibodies against the Abl family kinases c-Abl and Arg, Abi proteins, Src, and growth cone associated protein (GAP43). Sequential fractionation of the whole forebrain homogenate (H) yields the supernatant (P2 sup) and pellet fractions (P2) following low-speed centrifugation and growth cone particles (GC) and growth cone membrane fractions (GC Memb) following high-speed centrifugation through Ficoll gradient (Patterson and Skene, 1999). Abi, c-Abl, and Arg proteins appear enriched in the P2 and GC fractions over the P2 supernatant.

throughout development. Multiple bands corresponding to Abi-1 and Abi-2 proteins are observed by Western blot analysis of embryo and postnatal brain lysates. These results are consistent with previous work that has identified multiple splice variants for Abi-1 and Abi-2 (Wang *et al.*, 1996a; Biesova *et al.*, 1997; Taki *et al.*, 1998; Ziemnicka-Kotula *et al.*, 1998). Additionally, Abi proteins are modified posttranslationally by phosphorylation (Fig. 1E and Biesova *et al.*, 1997) and ubiquitination (Dai *et al.*, 1998). These modifications increase the complexity of the protein migration pattern associated with Abi expression.

The localization of *abi-1* and *abi-2* transcripts was identified throughout nervous system development. Our results show *abi-2* expression is most prominent in the CNS in embryos at E10 and E12 and throughout the CNS and in DRGs at E16. Like *abi-2*, Abl and Arg kinases are most highly expressed in the neuroepithelium at E9.5–10.25 (Koleske *et al.*, 1998). Although *abi-2* message is present throughout the developing neocortex at E16, it is more highly expressed in the region of postmigratory, differentiated neurons and glia that defines the cortical plate than in the migrating population of cells in the underlying intermediate zone or in the dividing neuroblasts of the ventricular zone (Angevine and Sidman, 1961). In contrast, *abi-1* does not show CNS enrichment in embryos and is not observed in DRGs. By postnatal day 0, *abi-1* and *abi-2* are highly expressed in similar regions of the brain, and this pattern is maintained in older mice. Sites of prominent *abi-1* and *abi-2* expression include the hippocampus, the dentate gyrus, the neocortex, the mitral cell layer of the olfactory bulb, and the Purkinje layer of the cerebellum. Abi-2 therefore may play a role in PNS development that is not shared by Abi-1, and Abi-1 and Abi-2 may have unique functions in early CNS development. The regional concentration of *abi-1* in the postnatal brain, not apparent in the embryo, suggests a late gestational or neonatal onset of possible neural cell-specific functions for Abi-1.

The localization of Abi-1 and Abi-2 within postmitotic neurons suggests that they may contribute to multiple processes in these cells. Expression of Abi-1 · EYFP and Abi-2 · EYFP in cultured neurons less than 1 week after plating yields a vesicular staining pattern that extends into neurites and is excluded from the nucleus. In neurons cultured for 2 weeks, Abi-1 · EYFP is predominantly somatodendritic and appears concentrated in a subset of dendritic spines. Abi1 · EYFP is also observed in the axon. Although transfection of fluorescent-tagged Abi-1 and Abi-2 into hippocampal cultures permitted observation of both Abi-1 and Abi-2 neuronal distribution *in vivo* in immature neurons, we were unable to successfully transfect more mature neurons with Abi-2 · EYFP. Perhaps Abi-2 protein is more unstable than Abi-1, or Abi-2 overexpression is toxic to these cells.

Abi-1 · EYFP and Abi-2 · EYFP distribute to multiple neuronal compartments. We wished to confirm this localization by examining endogenous Abi proteins using subcellular fractionation techniques. Following fractionation of neonatal and adult brains, endogenous c-Abl, Arg, and Abi proteins are observed in growth cone particles and at synaptic terminals. Abi proteins may therefore impinge upon Abl-family kinase functions in these structures. Studies in *Drosophila* have revealed effects of D-Abl during axonogenesis and growth cone pathfinding. *D-abl*$^{-}$/$^{-}$ flies exhibit premature arrest of

intersegmental nerve b (ISNb) growth cones leading to defective axon outgrowth (Wills *et al.*, 1999). Furthermore, additional *Drosophila* mutants have been characterized that link *D-abl* to axon outgrowth, including mutants for the D-Abl substrate enabled (Ena) and the actin binding protein Chickadee (Profilin) (Gertler *et al.*, 1995; Wills *et al.*, 1999).

Rac and other Rho-family GTPases are regulators of growth cone movement and directional guidance (Luo *et al.*, 1994, 1996; Threadgill *et al.*, 1997; Kaufmann *et al.*, 1998). Recent work has shown a role for Abi-1 in cytoskeletal reorganization mediated by Rac (Scita *et al.*, 1999). Microinjection of fibroblasts with antibodies to Abi-1 blocks membrane ruffling in response to PDGF (Scita *et al.*, 1999). Interestingly, the same phenotype is also observed in *abl*−/− cells (Plattner *et al.*, 1999). In the adult rat brain, *rac1* message is elevated in areas of synaptic plasticity, including the hippocampus, dentate gyrus, and granule and Purkinje cells of the cerebellum (Olenik *et al.*, 1997). *rac1* expression in the adult rat brain largely coincides with expression of *abi-1* and *abi-2* in the adult mouse brain. Thus, Abi proteins may colocalize with Rac1 in the brain and may participate in regulation of Rac function in neurons as has been proposed in fibroblasts (Scita *et al.*, 1999). Abi adaptors may integrate signaling pathways regulated by NRTKs and small GTPases at sites of dynamic cytoskeletal remodeling.

Our data suggest that Abi proteins are both pre- and postsynaptic. Sites of *abi-1* and *abi-2* enrichment in the postnatal brain correspond to projection neuron populations (e.g., mitral cells, cerebellar Purkinje cells) and regions which exhibit synaptic plasticity (Maness, 1992; Zhang *et al.*, 1997). Interestingly, recent work has shown an interaction between Abi-1 and the cytoskeletal protein erythroid spectrin (Ziemnicka-Kotula *et al.*, 1998). Erythroid-type brain spectrin has been shown to exhibit regulated binding to NMDA receptors, which are are key components of long term potentiation and synaptic plasticity (Wechsler and Teichberg, 1998). Future work will examine whether Abi proteins functionally interact with NMDA receptors.

Abi proteins undergo changes in phosphorylation and are shifted in reducing gels during post-natal brain development, suggesting Abi involvement in signaling events that may promote neuron differentiation and development. It is possible that the observed changes in Abi phosphorylation coincide with a decrease in the population of proliferating cells in the brain or with the attenuation of specific mitogenic signals. Abi-1 has previously been shown to become hyperphosphorylated on serine following mitogenic stimulation by serum or EGF treatment of serum-starved fibroblasts overexpressing EGFR (Biesova *et al.*, 1997). Significantly, we have recently shown that the c-Abl kinase is activated by the binding of the growth factors EGF and PDGF to receptor tyrosine kinases, and that this activation is mediated in part by Src-family kinases (Plattner *et al.*, 1999). Although we were unable to detect Abi tyrosine phosphorylation in brain lysates (data not shown), Abl-family kinases may participate in the generation of signals leading to Abi phosphorylation.

Although a number of proteins that interact with and are substrates for mammalian c-Abl or D-Abl have been proposed to contribute to Abl function in neural development, neuronal targets of Arg have not been previously identified (Van Etten, 1999). While c-Abl and Arg share significant sequence identity in the amino-termini, the large carboxy-terminal regions of these proteins are largely divergent. However, the site of Abi SH3 binding is conserved in the C-terminus of c-Abl and Arg (Perego *et al.*, 1991). Thus, Abi-1 and Abi-2 are excellent candidates for participating in c-Abl- and Arg-mediated events in neurons and neuronal precursors. Abl-family kinases may transduce signals from multiple receptors present in neuronal cell bodies, at synapses, or in growth cones. Abi proteins may serve as regulators or downstream targets of Abl-family kinases at each of these sites. Homozygous deletion of *abi-1* and *abi-2* in mice will enable us to further examine these possibilities. Studies are underway to ascertain the effects of loss of *abi-1* and *abi-2* on mouse neuronal development and function.

EXPERIMENTAL METHODS

Antibodies

Rabbit polyclonal anti-Abi-1 sera 6987 and 6988 were raised against the internal Abi-1 peptide HGNNQPARTGTLSRTNP. Rabbit polyclonal anti-Abi-2 antibody 7887 was raised against the internal Abi-2 peptide RFKVSTQNMKMGGLPRTTPPT. Rabbit polyclonal antibody anti-Abi 5421 has been described previously (Dai *et al.*, 1998). Monoclonal antibody 21-63 against c-Abl has been previously described (Schiff-Maker *et al.*, 1986). Mouse anti-Abl monoclonal antibody 8E9 was purchased from Pharmingen (San Diego, CA). Mouse monoclonal anti-phosphotyrosine antibody clone 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). A polyclonal antibody recognizing Arg was generously provided by Dr. A. Koleske (Department of Molecular Biophysics and Biochemistry, Yale

University, New Haven, CT). A polyclonal antibody recognizing c-Src and HRP-conjugated anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-GAP43 antibody was purchased from Boehringer-Mannheim (Indianapolis, IN). A monoclonal antibody recognizing synaptophysin was purchased from Sigma (St. Louis, MO). Rhodamine-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Pierce (Rockford, IL). HRP-conjugated Protein A was purchased from Amersham Pharmacia (Arlington Heights, IL).

Tissue Preparations

Tissue lysates for protein analysis were prepared as follows. Embryos from timed-pregnant CD-1 mice were dissected free of extraembryonic membranes and homogenized in ice cold RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) in the presence of protease and phosphatase inhibitors (10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4) (Gertler *et al.*, 1996). Brain extracts were similarly prepared. Lysates were clarified by centrifugation, and protein concentration was determined by the DC Protein Assay (Bio-Rad, Hercules, CA).

For *in situ* hybridization, embryos were prepared in one of two ways. Following dissection free of extraembryonic membranes, embryos either were immersed immediately in 2-methylbutane at −40°C or were fixed overnight at 4°C in 4% paraformaldehyde (PFA) prepared in phosphate-buffered saline (PBS). Fixed embryos were subjected to a sucrose gradient (10–30% sucrose in PBS) and embedded in 3.5% agar/3.5% sucrose and OCT. Sections of 16–25 μm thickness were cut on a Reichert Jung cryostat, mounted on CSS-100 silylated slides (Cel Associates, Houston, TX), and stored at −80°C until hybridization. Brains from postnatal mice were immersed in 2-methylbutane as described above or were dissected following perfusion of the mouse through the left ventricle of the heart with PBS followed by 4% PFA/4% sucrose. Following perfusion, brains were fixed and cryoprotected as described for embryos. Reagents used in preparation of tissues for *in situ* hybridization were pretreated with diethylpyrocarbonate (Sigma).

Synthesis of RNA Probes for abi-1 and abi-2

Abi-2 sense and antisense probes were prepared by *in vitro* transcription from a linearized construct of pBlueScript II Sk +/− plasmid (Stratagene, La Jolla, CA) into which a 435 nucleotide (nt) fragment of *abi-2* mouse genomic DNA was subcloned. This fragment included 48 nt of intronic sequence and 397 nt from a single exon encompassing the SH3 domain and a portion of the 3′ untranslated region (UTR) of murine *abi-2* (Dai *et al.*, 1998). *Abi-1* sense and antisense probes were prepared by *in vitro* transcription from either of two linearized templates. The first comprised a 373-nt cDNA fragment of murine *abi-1* spanning the SH3 domain and a portion of the 3′ UTR subcloned into pGEM-T (Promega, Madison, WI). The second was composed of the pGEM-T plasmid containing a 635 nt cDNA fragment of the 5′ end of murine *abi-1*.

In Situ Hybridization

In situ hybridization with [^{35}S]UTP-labeled probe (Dupont NEN, Boston, MA) was performed as described (Wang *et al.*, 1996b) with the following modifications. Following hybridization for 12–21 h at 50°C with 4000 cpm/μl [^{35}S]UTP-labeled probe, sections were washed briefly in room temperature 2× SSC and then for 1 h in 4 L of 2× SSC at 50°C with stirring. Subsequent RNase treatment and washes were carried out as described, with the exception that the final wash was continued overnight at room temperature following 3 h at 50°C. Slides were exposed to Kodak NT/B2 emulsion (Eastman Kodak, Rochester, NY) at 4°C for 2 weeks–1 month. Developed slides were counterstained with Hematoxylin-Eosin Y or methyl green. Most lower magnification images were acquired with a Leica M420 Photomakroskop M400 microscope with dark- and bright-field illumination and attached Diagnostic Instruments Spot Videocamera. Images were processed in Adobe Photoshop 5.0. Photography of some lower magnification images was performed with a Wild Photomakroskop M400 microscope with dark- and bright-field illumination using Kodak TMAX 100 film. Photographic negatives were scanned into Adobe Photoshop 4.0 with a Polaroid SprintScan 35 Plus scanner. Higher magnification images were captured by a Leitz Ortholux microscope with a Dage-MTI CCD 72S camera.

Analysis of Abl and Abi Proteins in Tissue Lysates

Western blot analysis to detect c-Abl or Abi proteins was carried out on 30 μg of tissue lysate resolved by sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS–PAGE) and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH). Anti-Abi serum 5421 and mouse monoclonal anti-Abl antibodies 21-63 or 8E9

were used. To examine specific expression of Abi-1 and Abi-2 proteins, lysates were incubated with anti-Abi-1-specific sera 6987 and 6988 or anti-Abi-2-specific serum 7887, respectively, for 6 h–overnight at 4°C. Lysates were subsequently incubated with protein A sepharose (PAS) beads (Amersham Pharmacia) for 1–1.5 h. Immunoprecipitates were washed twice with RIPA buffer in the presence of inhibitors (described above) and boiled in 2× SDS sample buffer. Proteins were resolved by SDS–PAGE and Western blot analysis was performed with anti-Abi-1 sera 6987 and 6988 or anti-Abi serum 5421.

To determine the phosphorylation status of Abi proteins in the mouse brain, lysates (2 mg total protein) were incubated for 2 h–overnight at 4°C with anti-Abi serum 5421, anti-Abi-1 sera 6987 and 6988, anti-Abi-2 serum 7887, or normal rabbit serum. Following incubation for 30 min–1 h with PAS beads, immunoprecipitates were washed extensively with RIPA buffer and inhibitors, followed by potato acid phosphatase (PAP) buffer (40 mM Pipes, pH 6.0, 1 mM DTT, 1 mM $MgCl_2$, 10 μg/ml aprotinin). Immunoprecipitates bound to PAS beads were resuspended in PAP buffer in the presence or absence of 2 μg potato acid phosphatase (Boehringer Mannheim) as indicated, and incubated 10 min at 30°C. Following an additional wash with RIPA buffer and inhibitors, PAS beads were boiled with 2× SDS sample buffer and electophoresed on SDS–PAGE gels. Subsequent Western analysis was performed as described above.

Cell Culture and Transfection

Embryonic day-18 (E18) rat hippocampal tissue (BrainBits, Springfield, IL) was dissociated by gentle trituration and cells were plated on poly-d-lysine (50 mg/ml)-coated coverslips at a density of 23×10^3 cells/cm^2. Cells were grown in Neurobasal medium (Gibco-BRL), complemented with B27, 0.5 mM glutamine, and 25 mM l-glutamate, and maintained at 37°C in 5% CO_2. The same medium (minus l-glutamate) was used for partial medium exchanges every 4 days. Hippocampal cells were transfected on the days indicated, using FuGENE 6 Reagent (Boehringer-Mannheim) as described in the package insert. In short, 2 μg of plasmid DNA was mixed with 4 μl of FuGENE 6 Reagent diluted in 100 μl of Neurobasal medium. This mixture was incubated at room temperature for 15 min and then added to the culture medium. Fluorescence images were obtained using a Micromax CCD camera (Princeton Electronics) attached to a Nikon Diaphot epi-fluorescence microscope equipped with a FITC filter set (Chroma Technology Corp., Brattleboro, VT).

Enhanced Yellow Fluorescent Protein (EYFP) Fusions

The Abi-1 coding region, minus the first methionine, and 123 nt of 3′ UTR were amplified by PCR and subcloned into the plasmid pEYFP-C1 (Clontech, Palo Alto, CA) at the *Bgl*II site. The isoform of Abi-1 used to construct the Abi-1 · EYFP fusion was obtained from EST zr24a06.r1 (GenBank Accession No. AA232072, ATCC, Rockville, MD) (R. C. Quackenbush, and A. M. Pendergast, unpublished results). The Abi-2 coding region was amplified by PCR and subcloned into pEYFP-C1 at the *Bam*HI site. The isoform of Abi-2 used to construct the Abi-2 · EYFP fusion was obtained from EST yo44f11.s1 (GenBank Accession No. R87714, ATCC) (Z. Dai and A. M. Pendergast, unpublished results). EYFP was located at the N terminus of both constructs.

Subcellular Fractionation of Neonatal and Adult Rat Brain Lysates

Neonatal (P3) rat brain fractions to yield growth cone particles were generously provided by Dr. J. H. P. Skene (Department of Neurobiology, Duke University Medical Center, Durham, NC) and were prepared as described (Patterson and Skene, 1999). Adult rat brain postnuclear supernatant and synaptosome fractions were the generous gift of Dr. R. Colbran (Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN) and were prepared as published (Strack *et al.*, 1997).

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