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Mena Is Required for Neurulation and Commissure Formation

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Summary

Mammalian enabled (Mena) is a member of a protein family thought to link signal transduction pathways to localized remodeling of the actin cytoskeleton. Mena binds directly to Profilin, an actin-binding protein that modulates actin polymerization. In primary neurons, Mena is concentrated at the tips of growth cone filopodia. Mena-deficient mice are viable; however, axons projecting from interhemispheric cortico-cortical neurons are misrouted in early neonates, and failed decussation of the corpus callosum as well as defects in the hippocampal commissure and the pontocerebellar pathway are evident in the adult. Mena-deficient mice that are heterozygous for a Profilin I deletion die in utero and display defects in neurulation, demonstrating an important functional role for Mena in regulation of the actin cytoskeleton.

Introduction

In recent years, much has been learned about the signals that guide axons as they navigate toward their targets. It is known that axonal growth cones respond to a variety of attractive and repulsive cues present in the extracellular environment and that response to such cues is often modulated by phosphorylation-dependent signaling (Caroni, 1998; Flanagan and Van Vactor, 1998). The identities of the downstream targets of these signaling events and how they ultimately transduce signals into effects on growth cone motility remain unclear.

Genetic screens in Drosophila and C. elegans have identified cell surface receptors that regulate growth cone behavior at particular choice points and/or in response to chemoattractants and repellents. For example, the Robo/Sax3 receptor family controls a repulsive response to midline signals (Kidd et al., 1998; Zallen et al., 1998), while Netrins and their receptors have been implicated in both repulsive and attractive axon guidance signaling along the dorsoventral axis in C. elegans, in motor and commissural neurons in Drosophila, and in commissural axons in vertebrates (Keynes and Cook, 1996).

The Abl nonreceptor tyrosine kinase has also been implicated in axon guidance in Drosophila. The requirements for Abl in axon formation are more obvious when combined with mutations in one of a number of loci that act as dose-dependent modifiers of Abl (Gertler et al., 1993). The mammalian homolog of one of these modifiers, Disabled (Dab), is required for proper control of neuronal cell migration in the developing cortex (Howell et al., 1997). Genetic screens for suppressors of Abl-dependent phenotypes identified multiple alleles of only one locus, Enabled (Ena; Gertler et al., 1990). Heterozygosity for Ena alleviates Abl-dependent neuronal phenotypes, while homozygosity for Ena alone causes highly penetrant defects in axon guidance and fasciculation (Gertler et al., 1995).

Ene is the prototype of a family of proteins that includes Mena, VASP, and EVL (the “Ena/VASP” family). Mena (mammalian enabled) was identified as a mammalian ortholog of Drosophila Ena (Gertler et al., 1996). VASP (vasodilator-stimulated phosphoprotein) was originally characterized in platelets as a stoichiometric substrate of cyclic nucleotide-dependent kinases and is required for the regulation of platelet aggregation, a process that depends upon rapid actin assembly (Halbrügge and Walter, 1989; Aszodi et al., 1999). EVL (Ena-VASP-like) was identified as an expressed sequence tag with homology to Ena and VASP (Gertler et al., 1996).

The Ene/VASP family shares a common structural organization composed of highly conserved NH2- and COOH-terminal domains called Ena/VASP homology 1 and 2 (EVH1 and EVH2) that flank a central proline-rich domain. The EVH1 domain mediates subcellular targeting by binding to the motif D/EFPPPP, which is found in the cellular focal adhesion proteins Zyxin and Vinculin and in the ActA protein from the intracellular bacteria Listeria monocytogenes (Niebuhr et al., 1997). The EVH2 domain contains a predicted coiled coil-like sequence and is thought to mediate oligomerization of Ene/VASP proteins (Ahern-Djamali et al., 1998). The central proline-rich domain mediates direct interactions with the actin-binding protein Profilin and with the SH3 domains of the tyrosine kinases Abi, Arg, and Src (Gertler et al., 1996). A higher molecular weight form of Mena is produced by the alternate inclusion of an exon (the “+” exon) between the EVH1 domain and the proline-rich core; this Mena(+) isoform is enriched in the developing nervous system and is the only Mena isoform found to be tyrosine phosphorylated during development (Gertler et al., 1996). Mena, VASP, and EVL share...
a single conserved site for phosphorylation by cyclic nucleotide-dependent protein kinase A (PKA) and are in vivo substrates for PKA (Butt et al., 1994; Gertler et al., 1996; F. B. G., unpublished data). In addition, Mena and VASP share a second consensus phosphorylation site that, in the case of VASP, has also been shown to be a target for cyclic GMP-dependent kinases (Butt et al., 1994).

Several findings implicate Mena and VASP in the regulation of cytoskeletal dynamics. First, Mena and VASP accumulate at focal adhesions, which are sites of bidirectional signaling between the cytoskeleton and the extracellular matrix, and in regions of dynamic actin remodeling such as the lamellipodia at the leading edge of motile cells (Reinhard et al., 1992; Gertler et al., 1996). Second, at least one member of the Ena/VASP family must be present for rapid, directed, actin-based movement of Listeria (V. Laurent, J. Wehland, F. B. G., and M. Carlier, unpublished data). Profilin has also been implicated in Listeria movement (Theriot et al., 1994), and it has been postulated that one function of Mena and VASP in this context is to bind ActA and link Profilin to the surface of Listeria. Third, expression of the neural specific Mena(+) isoform in fibroblasts leads to the formation of actin-rich cell surface protrusions, suggesting that at least this Mena variant can induce actin remodeling (Gertler et al., 1996). Ectopic expression of the other form of Mena, or of EVL or VASP, does not induce actin protrusion (Gertler et al., 1996; F. B. G., unpublished data).

The fact that Mena(+) is highly expressed in the developing nervous system and can induce actin protrusion led to the hypothesis that, like Ena, Mena(+) could play a role in regulating growth cone motility and axon guidance. In the developing axon, actin polymerization drives formation of growth cone filopodia and is required for axon pathfinding, whereas microtubule polymerization extends and stabilizes the axon shaft once a direction is chosen (Mitchison and Kirschner, 1988). Extension or retraction of filopodia is the first morphological change observed in response to guidance signals, suggesting that proteins that regulate actin polymerization in the filopodia may play a central role in transducing extracellular signals into changes in growth cone motility.

Although many proteins have been reported to be in growth cones, relatively few have been shown to be enriched in filopodia. Among those identified are β1 integrin, a transmembrane protein that binds extracellular ligands and is physically linked to the actin cytoskeleton, and members of the Ezrin-Radixin-Moesin (ERM) family, intracellular proteins that physically link actin filaments to the plasma membrane (Wu et al., 1996). Under certain conditions, phosphotyrosine is also detected at the distal tips of filopodia, although the identity of the tyrosine-phosphorylation substrate(s) has not been determined (Wu and Goldberg, 1993). Localization of these proteins in the filopodia appears to affect growth cone dynamics and may be important in regulating axon guidance (Wu and Goldberg, 1993; Wu et al., 1996; Paglini et al., 1998).

In this report, we present data indicating that Mena plays an important role in the regulation of growth cone dynamics and axon guidance. We have generated Mena-deficient mice and analyzed the phenotype of the mutant animals. We show that Mena is expressed in the developing nervous system and is required for the normal formation of several major axonal projection pathways in the brain, including the corpus callosum and hippocampal commissure. Consistent with a role for Mena in axon guidance, we find that Mena is highly concentrated in the distal tips of growth cone filopodia. Finally, we describe a potent interaction between mutations in Mena and Profilin I that reveals a role for these molecules in neural tube closure and provides the first genetic evidence linking Mena function to regulation of actin cytoskeletal dynamics.

Results

Generation and Biochemical Characterization of Mena-Deficient Mice
A targeted disruption of the Mena locus was generated through homologous recombination in embryonic stem
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Figure 2. Western Blot Analysis of Mena Expression Patterns

(A) Detection of Mena, EVL, and VASP in extracts from adult brain and organs.

(B) Detection of Mena in extracts from adult brain regions. Extracts from glial and cortical cultures are shown to demonstrate that the 140 kDa form of Mena is neuron-specific. Brain regions include: olfactory bulb, hippocampus, striatum (caudate, putamen, and globus pallidus), cortex, midbrain (including thalamus), pons/medulla, and cerebellum. Extracts from total brain of wild-type (+/+), heterozygous (+/−), and homozygous (−/−) adult animals are shown for comparison.

(C) Detection of Mena, EVL, and VASP in the developing brain. Extracts were prepared from either total brain (E11-E16) or from the indicated brain regions (E18, P1, P10). Extracts in (C) were prepared from total E13 heads, while extracts in Figure 2C were prepared from brain only, indicating that at early stages (E13) EVL expression in the head is in regions outside the brain. Analysis of Tubulin levels confirmed that equal amounts of protein were loaded in each lane (data not shown).

Mena Protein Expression

The distribution of Mena in wild-type adult organs was compared to that of EVL and VASP (Figure 2A). The 140 kDa form of Mena was detected only in the brain, while the 80 kDa form of Mena was expressed predominantly in brain, testis, ovaries, and fat. In contrast, EVL and VASP were most highly expressed in thymus and spleen, and the relative intensities of the phospho and dephospho forms varied from tissue to tissue, suggesting that EVL and VASP may be differentially regulated in the brain and organs. Because Mena was expressed at high levels in the brain, while both VASP and EVL were expressed at low levels, the distribution of Mena in adult and developing brain was characterized in greater detail.

The 80 and 140 kDa forms of Mena were detected in all regions of the adult brain, with highest levels in the hippocampus, cortex, and midbrain, and lowest levels in the striatum and cerebellum (Figure 2B). The 140 kDa form of Mena was expressed at relatively high levels in serum-free cortical cultures (which are enriched for neurons) and was not detected in glial cultures, suggesting the 140 kDa form is indeed neuron-specific and that it may be the predominant form of Mena in neurons. In embryonic brains, all three forms of Mena are detected at embryonic day 11 (E11), the earliest time point examined (Figure 2C). Expression of the 88 kDa form decreased steadily and became almost undetectable by

(ES) cells. Exons 2 and 3 of the Mena gene were replaced by a cassette encoding a β-galactosidase (LacZ)-neomycin fusion protein (βgeo; Figure 1A). The resulting locus produced a fusion protein containing the 5' untranslated region of Mena and the initiator methionine fused in frame to βgeo. This strategy resulted in a Mena protein null mutant and introduced a lacZ reporter gene under the control of the endogenous Mena promoter (creating the Menaβgeo allele).

Four independent Menaβgeo/−ES cell lines were used to obtain germline transmission of the mutant allele. Genotypes of the resulting animals were determined by Southern blot analysis (data not shown) and polymerase chain reaction (PCR; Figure 1B). All four ES cell lines gave equivalent results, so one of these lines was selected for further analysis. Mena homozygous mutant animals (Menaβgeo/βgeo) were fully viable and were recovered in the appropriate Mendelian ratios (data not shown).

Homozygous mutant animals were smaller than their littermates until adulthood and exhibited abnormal cage behavior, including reduced activity (data not shown).

Western blot analysis of extracts from embryonic heads and adult brains was used to verify that the Menaβgeo allele was a protein null. As previously reported, three forms of Mena (corresponding to bands of 80, 88, and 140 kDa; Gerster et al., 1996) are expressed in embryonic heads, while only the 80 and 140 kDa forms are expressed in adult brains (Figure 1C). Expression of all three forms of Mena was reduced in the heterozygotes and completely eliminated in the homozygotes. Finally, reduction in Mena expression may lead to a slight increase in expression of EVL and VASP (Figure 1C; The

52 and 50 kDa bands of EVL and VASP represent serine/threonine phosphorylated versions of the 48 and 46 kDa bands, respectively; Halbrügge and Walter, 1989; A. Lambrechts and F. B. G., unpublished data).
was determined by in situ hybridization of whole-mount 129sv (Wahlsten, 1982). Therefore, the Mena scripts or a probe specific to the Mena (adult brain (Figure 2C). Spontaneous agenesis of the corpus callosum has been to be fairly constant throughout development of the sure and the hippocampus, including the dentate gyrus, pressed in the branchial and pharyngeal arches, but not the forebrain lateral ventricles (Probst bundles [P]; Figure 3E). In agreement with anti-Mena Western blot data (Figures 2B and 2C), relatively low level expression was detected in the striatum and globus pallidus. Double labeling of sections with LacZ and antibodies to either neurofilament protein, MAP-2 or Py, an antigen found in a subset of projection neurons (Woodhams et al., 1989), confirmed that in the cortex Mena was expressed in pyramidal neurons of layers 2/3 and 5 (Figures 4C - 4H; MAP2 data not shown).

Axonal Pathfinding Defects in Mena\(^{bgeo}\)/\(^{bgeo}\) Mutant Animals

Given Mena expression patterns and the axonal defects detected in Ena mutant flies (Gertler et al., 1995), we speculated that Mena\(^{bgeo}\)/\(^{bgeo}\) homozygous animals would display abnormalities in the brain. Indeed, neurofilament staining of sections through the forebrains of adult Mena\(^{bgeo}\)/\(^{bgeo}\) animals revealed striking abnormalities in the corpus callosum, the major axonal projection pathway connecting the two hemispheres of the brain (Figure 5D). Wild-type littermates showed a morphologically normal corpus callosum, as did Mena\(^{bgeo}\)/\(^{bgeo}\) heterozygotes (Figure 5A). In Mena\(^{bgeo}\)/\(^{bgeo}\) animals, fibers in the corpus callosum appeared to reach a point just medial to the cingulum bundle as normal but then failed to project medially and cross the midline. Instead, most of the fibers formed dense neuromas just dorsomedial to the cingulum bundle (Probst bundles [P]; Figure 5D; Probst, 1901). In contrast, the anterior commissure and the hippocampus, including the dentate gyrus, appeared to develop normally (Figures 5E and 5F).

Spontaneous agenesis of the corpus callosum has been observed in several inbred strains of mice, including 129sv (Wahlsten, 1982). Therefore, the Mena mutation was backcrossed to C57BL6 mice for ten generations, embryos using either a probe that detects all Mena transcripts or a probe specific to the Mena(+) exon. At E8.5, Mena was particularly enriched in the neuroepithelium (single arrows), and the cephalic neural folds overlying the forebrain region (double arrow). Low level expression is detected in the branchial arch (ba) and the forelimb bud (flb). (B) Dorsal view of the same E8.5 specimen in (A) shows high level of Mena expression in the dorsal neural crest (arrow head). (C) E10.5 dorsal view. High level Mena expression is detected in the somites (S) and dorsal root ganglia (DRG). (D) Side view of the same E10.5 embryo in (C). Mena expression is detected in the forebrain (fb), midbrain (mb), branchial arch (ba), pharyngeal arch (pa), forelimb bud (flb), hindlimb bud (hfb), and somites (S). (E) A probe specific for the (+) exon detects expression of the neuronal specific 140 kDa form of Mena in the developing central nervous system, including the forebrain (fb) and midbrain (mb), but not in the DRG or limb buds.

E16, while expression of the 140 kDa form began to increase at E13 and peaked between E16 and E18. In contrast to Mena, EVL expression in the brain was first detected at E15 (Figure 2C). In situ hybridization confirmed that in early stage embryos EVL is highly expressed in the branchial and pharyngeal arches, but not in the brain (data not shown). VASP expression appeared to be fairly constant throughout development of the brain, but then decreased to relatively low levels in the adult brain (Figure 2C).

The pattern of Mena expression in embryonic tissues was determined by in situ hybridization of whole-mount embryos using either a probe that detects all Mena transcripts or a probe specific to the Mena(+) exon. At E8.5, Mena was particularly enriched in the neuroepithelium, the forebrain, and the somites (Figure 3A). A dorsal view of the same embryo shows that Mena was highly expressed in the edges of the neural folds (Figure 3B). By E10.5, Mena expression was detected in the brain, dorsal root ganglia (DRG), somites, and limb buds (Figures 3C and 3D). In addition, Mena was highly expressed in the branchial and pharyngeal arches, neural crest-derived structures that give rise to portions of the face and neck. At E10.5, a probe for the (+) exon detected the Mena(+) isoform in regions of the developing central nervous system (CNS), but not in the DRG of the developing peripheral nervous system (Figure 3E).

The Mena\(^{bgeo}\) allele provides convenient and sensitive means to characterize Mena expression at the cellular level. bgeo activity is restricted to cell bodies and can be used to identify cell types, but it is not readily detected in axonal projections. High levels of Mena\(^{bgeo}\) expression were detected in distinct bands of cells in the developing cortex at E16, a time when neurons are migrating from the ventricular zone to the cortical layers and axons are beginning to project across the corpus callosum (Figure 4A; Macklis, 1993; Koester and O’Leary, 1994). In the adult brain, Mena\(^{bgeo}\) expression was detected in laminae 2/3 and 5 of the cortex and was particularly enriched in the hippocampus and the septum (Figures 4C and 4F). The pattern of Mena expression in embryonic tissues appeared to develop normally (Figures 5E and 5F). Wild-type littermates showed a morphologically normal corpus callosum, as did Mena\(^{bgeo}\)/\(^{bgeo}\) heterozygotes (Figure 5A). In Mena\(^{bgeo}\)/\(^{bgeo}\) animals, fibers in the corpus callosum appeared to reach a point just medial to the cingulum bundle as normal but then failed to project medially and cross the midline. Instead, most of the fibers formed dense neuromas just dorsomedial to the cingulum bundle (Probst bundles [P]; Figure 5D; Probst, 1901). In contrast, the anterior commissure and the hippocampus, including the dentate gyrus, appeared to develop normally (Figures 5E and 5F).

Spontaneous agenesis of the corpus callosum has been observed in several inbred strains of mice, including 129sv (Wahlsten, 1982). Therefore, the Mena mutation was backcrossed to C57BL6 mice for ten generations, and all mice analyzed in these experiments were the coisogenic F1 progeny of crosses between the 129sv inbred and the C57BL6 backcrossed lines. Agenesis of the corpus callosum was observed in 11 out of 20 Mena\(^{bgeo}\)/\(^{bgeo}\) animals but was never observed in 20 littermate control animals, indicating that the defects result from loss of Mena rather than as an effect of genetic background. The penetrance of commissural defects in the Mena mutants is similar to that seen in several other genetic models for axon guidance (e.g., Orioli et al., 1996) and may reflect redundancy in the Ena/VASP family (see Discussion). It is also possible that the remaining
Mena<sup>bgeo</sup>/<sup>bgeo</sup> mutants have more subtle defects in midline crossing, the identification of which will require further analysis.

Using silver staining to visualize the fiber tracts at higher resolution, it was possible to observe that in the Mena<sup>bgeo</sup>/<sup>bgeo</sup> mutants a few fibers emerged from the Probst bundles and projected medially, crossing the midline just above the dorsal fornix (Figures 5J - 5L). Within the fornix, fibers of the hippocampal commissure appeared abnormal; instead of crossing contralaterally, they appeared to reach the midline and project ipsilaterally (compare Figures 5I and 5L). In more caudal sections, hippocampal commissure fibers crossed the midline (data not shown), indicating that the defects in the hippocampal commissure are most likely due to misrouting and/or reduction in the number of fibers. Close examination of the sections revealed the presence of cells at the midline (Figure 5L), indicating that the interhemispheric fissure had fused properly during development. Defects in midline fiber crossing were also observed in the pons, where decreased numbers of pontocerebellar fibers reached and crossed the midline (Figures 5M and 5N). No defects were observed in other commissures, including spinal motor neuron tracts, or in cortical lamination, indicating that there was not a global failure in midline crossing or neuronal cell migration (data not shown). DARPP-32 immunocytochemistry was used to analyze axonal projections in the internal capsule for potential defects in axonal fasciculation and/or pathway formation; however, no obvious disturbances were noted in these axonal pathways (data not shown).

To determine if the corpus callosum failed to form during development or whether it formed and then degenerated, we analyzed the development of this structure by Dil labeling. At P0, the corpus callosum of wild-type animals was well formed and projected contralaterally through the midline (Figures 6A and 6B). In Mena<sup>bgeo</sup>/<sup>bgeo</sup> littermates, fibers of the corpus callosum reached the presumptive cingulum bundle but then appeared to project dorsally and turn away from the midline (Figures 6C and 6D). Therefore, the defects in the corpus callosum associated with the Mena<sup>bgeo</sup>/<sup>bgeo</sup> mutation were due primarily to a failure of the axons to project across the midline during development.

Mena Localization in Neuronal Growth Cones
Given the axon guidance defects in the Mena mutants, it was important to determine if the subcellular distribution of Mena was consistent with a role for Mena in axon guidance and/or growth cone motility. To do this, we chose to use cultured primary embryonic hippocampal neurons, which elaborate multiple dendrites and a single morphologically and histologically distinguishable axon (Goslin and Banker, 1991). Immunocytochemical analysis revealed that Mena was highly enriched in the lamellapodium and at the tips of the axonal growth cones (Figure 7A). Identical results were observed with poly- and monoclonal anti-Mena antibodies; no signal was observed when primary antibodies were omitted (data not shown). Similar Mena localization was seen in dendritic growth cones and at various stages of differentiation (data not shown), suggesting that Mena may function in both types of growth cones throughout development.
Figure 5. Histological Comparison of Wild-Type and Mutant Adult Brains

Matched section of wild-type and homozygous mutant (Mena<sup>bgeo/bgeo</sup>) brains were analyzed either by neurofilament immunocytochemistry (A-F) or by silver staining (G-N).

(A-C) Neurofilament staining of rostral to caudal sections through a wild-type brain reveals the properly developed corpus callosum (cc), cingulum bundle (cng), anterior commissure (ac), and hippocampal regions CA1, CA3, and dentate gyrus (DG).

(D-F) Similar sections through a homozygous mutant reveal the presence of Probst bundles (P) and failed midline crossing of axons traversing the corpus callosum.

(G-L) Silver staining of matched sections through wild-type (G-I) and Mena<sup>bgeo/bgeo</sup> mutant (J-K) brains at magnification of 2× (G and J), 4× (H and K), and 20× (I and J).

(G-I) In the wild-type brain, it is possible to see the pre- and postcommissural fornix (fo<sub>p</sub> and fo<sub>q</sub>, respectively) and the hippocampal commissure (hc), in addition to those structures seen by neurofilament staining.

(J-L) In the Mena<sup>bgeo/bgeo</sup> mutant, many structures are abnormal or missing.

(K and L) A few fibers from the corpus callosum appear to project ventrally and cross just above the dorsal fornix (arrow head).

(L) Cells are visible in the midline (arrow), but fibers of the hippocampal commissure do not appear to cross as they do in the wild-type control (compare to [I] and [L]).

(M and N) Comparison of matched sections through the pons of wild-type (M) and Mena<sup>bgeo/bgeo</sup> mutant (N) brains reveals that a decreased number of axons reach and eventually cross the midline.

The pattern of staining was the same when antiserum specific for the (+) exon was used, suggesting that the neuron-specific 140 kDa form of Mena is enriched at the tips of filopodia (data not shown). Whether Mena staining in filopodia is due solely to the presence of the 140 kDa form, or whether both the 80 and 140 kDa forms are present in filopodia, remains to be determined. To put Mena localization in the context of other proteins that have been localized to growth cone filopodia, triple labeling was done to localize Mena, ERM proteins, and filamentous actin (F-actin; Figure 7B). The merged image clearly demonstrates that in filopodia, Mena staining is observed distal to both actin and the ERM proteins.

Genetic Interaction between Mena and Profilin I

A key to understanding Mena function comes from biochemical studies of Mena ligands and of Mena function in the actin-based motility of Listeria. Mena binds with high affinity to Profilin I (Gertler et al., 1996), an actin-binding protein that plays a role in regulating the rate of actin polymerization (Theriot and Mitchison, 1993). Both Ena/VASP proteins and Profilin are required for
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Figure 6. Analysis of Axonal Projections in the Developing Brain

Axonal projections were viewed by Dil dye tracing in matched sections through the corpus callosum of P0 wild-type (A and B) and Mena<sup>geo/geo</sup> mutant (C and D) brains. (A and C) Low magnification bright field images were traced (shown as a white line) and overlaid on the fluorescence image in order to show orientation. The boxed area is shown at higher magnification (B and D). Arrows indicate the position of the midline. Arrowheads (D) point to misrouted axons.

Figure 7. Mena Localization in Growth Cone Filopodia

Embryonic hippocampal neurons were fixed and labeled with antibodies to detect Mena and ERM proteins and with rhodamine-phalloidin to detect filamentous actin (F-actin).

(A) Mena (green) is enriched at the tips of growth cone filopodia, distal to F-actin (red).

(B) Triple labeling reveals that Mena (red) is distal to both F-actin (green) and the ERM proteins (blue). This is most clearly seen in the merged image.

rapid movement and cell-to-cell spread of the Listeria (Smith et al., 1996; Niebuhr et al., 1997). These observations led to the hypothesis that one function of Mena may involve its ability to bind Profilin, which could in turn modulate actin dynamics. We used a genetic approach to test the significance of the Mena-Profilin I interaction, reasoning that reducing the amount of Profilin I within cells might sensitize animals to loss of Mena and thereby expose requirements for these proteins. In an otherwise wild-type animal, profilin I heterozygotes are viable, but produce 50% of the normal amount of Profilin I, while profilin I homozygous mutants display preimplantation lethality (W. W. and D. K., unpublished data). Animals doubly heterozygous for Mena and profilin I mutations were mated, and the genotypes of viable progeny classes were determined (Table 1). Strikingly, no viable Mena homozygous/profilin I heterozygous (Mena<sup>geo/geo</sup>;profilin I<sup>+/−</sup>) animals were recovered, while other progeny types, including Mena<sup>geo/geo</sup>;profilin I<sup>−/−</sup>, were recovered at the expected frequency. No significant changes in profilin levels were detected in Mena<sup>geo/geo</sup> animals (data not shown). Genotype analysis at E9.5 and E16 revealed that Mena<sup>geo/geo</sup>;profilin I<sup>−/−</sup> embryos were present in Mendelian frequencies (data not shown), suggesting that these animals die perinatally.

Light microscopy of E9.5 embryos revealed that the Mena<sup>geo/geo</sup>;profilin I<sup>+/−</sup> animals were smaller than their Mena<sup>geo/geo</sup>;profilin I<sup>+/+</sup> littermates and often had abnormally formed heads (Figures 8A and 8B). Analysis at E9.5 indicated that the cephalic neural tube failed to close in half (6 of 13) of the Mena<sup>geo/geo</sup>;profilin I<sup>−/−</sup> embryos, but was closed in all other embryos (n = 104; Figures 8C and 8D). Cephalic neural tube closure is initiated at four distinct points (Copp, 1994). Close analysis of the Mena<sup>geo/geo</sup>;profilin I<sup>−/−</sup> animals revealed that...
the defects in neural tube closure occurred at closure points 1, 2, and 4 (3 of 13 embryos) or at points 2 and 4 (3 of 13). At E16.5, failure of neural tube closure sometimes manifested as either exencephaly (4 of 9 embryos) or anencephaly (1 of 9 embryos). Consistent with their apparent role in neural tube closure, Mena and Profilin I are both highly expressed in cephalic neuroectoderm (Figures 3A and 3B; A. Lambrechts and F. B. G., unpublished). These results indicate that a 50% reduction in the concentration of profilin I sensitizes animals to a loss of Mena and suggest a requirement for Mena and Profilin I function in neural tube closure, an actin-dependent process.

Discussion

Mena Is Required for Commissural Axon Guidance

A great deal of interest has focused on elucidating the cytoskeletal basis of axonal growth cone motility and guidance. Our results indicate that Mena, a molecule known to be involved in actin-cytoskeletal dynamics, is required for the formation of several major axonal projection pathways in the brain. Mice lacking Mena are viable but show striking malformation of the corpus callosum, hippocampal commissure, and the pontocerebellar fibers. The morphology of other axonal pathways, including the anterior commissure and spinal motor neurons, appeared normal, indicating that Mena is not required for formation of these pathways. Dye labeling experiments revealed that the callosal axons appear to be misrouted during development. These data, in conjunction with the finding that Mena is highly enriched at the distal tips of growth cone filopodia, suggest that Mena plays a critical role in commissural axon guidance.

Studies of invertebrate Mena homologs also suggest a role for Mena in axon guidance. Ena, the Drosophila homolog of Mena, was originally identified as a suppressor of CNS defects in abl/dab mutants (Gertler et al., 1990). Ena homozygotes show highly penetrant CNS and motor axon guidance defects, and neural specific expression of Ena greatly attenuates the motor neuron phenotype in Ena mutant embryos (Gertler et al., 1995; Wills et al., 1999a [this issue of Neuron]). Furthermore, mutations in Ena suppress the CNS axon guidance defects associated with the abl/fasciclin I double mutant (Elkins et al., 1990; Gertler et al., 1990). Finally, the C. elegans Unc34 locus, which is required for the guidance of certain axons, was recently identified as a member of the Ena/VASP family (Garriga and B. G., unpublished data).

While we favor a model in which Mena function is required within the axons for guidance, the present data do not permit us to exclude the possibility that the axonal phenotypes in Mena mutants arise as a secondary consequence of other defects, such as failure to form the “glial sling” (a band of glial cells that support axons as they cross the midline [Silver et al., 1982]). Several observations lead us to believe, however, that the defects in commissural axon guidance in Mena mutants are cell autonomous. First, dye labeling experiments indicated that in Mena mutants axons of the developing corpus callosum appear to turn laterally and project away before contacting the midline. In cases of callosal agenesis associated with glial sling defects, callosal axons are reported to contact the meninges at the midline.

Table 1. Genotypes of Progeny from Mena<sup>+/−</sup> profilin I<sup>+/−</sup> × Mena<sup>+/−</sup> profilin I<sup>+/−</sup> Matings<sup>*</sup>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mena&lt;sup&gt;+/−&lt;/sup&gt; profilin I&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>Mena&lt;sup&gt;−/−&lt;/sup&gt; profilin I&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>Mena&lt;sup&gt;+/−&lt;/sup&gt; profilin I&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Mena&lt;sup&gt;−/−&lt;/sup&gt; profilin I&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Mena&lt;sup&gt;+/−&lt;/sup&gt; profilin I&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Mena&lt;sup&gt;−/−&lt;/sup&gt; profilin I&lt;sup&gt;+/+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency</td>
<td>Expected&lt;sup&gt;#&lt;/sup&gt;</td>
<td>1/12</td>
<td>1/6</td>
<td>1/12</td>
<td>1/6</td>
<td>1/3</td>
</tr>
<tr>
<td># Expected</td>
<td>28</td>
<td>56</td>
<td>28</td>
<td>56</td>
<td>112</td>
<td>28</td>
</tr>
<tr>
<td># Observed</td>
<td>38</td>
<td>72</td>
<td>38</td>
<td>67</td>
<td>121</td>
<td>0</td>
</tr>
</tbody>
</table>

*Genotypes were determined between postnatal day 8 and 10. Mena<sup>+/+pro</sup> is abbreviated as Mena<sup>+/+</sup>; Mena<sup>−/−</sup> is abbreviated as Mena<sup>−/−</sup>.<sup>#</sup>profilin I<sup>+/−</sup> is preimplantation lethal, therefore progeny classes containing the profilin I<sup>−/−</sup> genotype were omitted from the calculation of expected frequency.
before turning and projecting ipsilaterally (Ozaki and Wahlsten, 1993). Second, in the Mena mutants, defects were also observed in the pontocerebellar pathways, the formation of which is not known to be dependent on the glial sling. Third, in some of the mutant animals, a few callosal fibers were able to cross the midline, suggesting that during development there is a substrate within the midline that is capable of supporting fiber crossover. While these observations are not definitive proof of cell autonomy, it is striking that mutations in Mena or its homologs result in apparent axon guidance defects in three different organisms, especially since the issue of glial sling formation does not appear to apply to the invertebrate homologs.

The neural tube closure defects seen in the Mena<sup>geo</sup>/geo; profilin I<sup>b</sup> animals reveal that Mena plays a critical role in neurulation in addition to its function in axon guidance. Mena function in neurulation involves Profilin and therefore might be linked to regulation of the actin cytoskeleton. Cephalic neural tube closure depends on actin-driven changes in the shape of cells within the dorsal-lateral hinge point (DLHP) of the neuroepithelium (Karfunkel, 1971). During the process of convergence, the cells of the DLHP become elongated and wedge shaped, providing the motive force for dorsolateral furrowing (Smith and Schoenwolf, 1997). The phenotypes of the Mena<sup>geo</sup>/geo; profilin I<sup>b</sup> embryos indicate a failure of neural tube closure sometime between convergence and fusion of the neural folds and may result from improper regulation of actin dynamics within the DLHP. Furthermore, it seems likely that Mena and Profilin I may be involved in regulating actin dynamics in many cell types throughout development, but the severe neurulation defects in the Mena<sup>geo</sup>/geo; profilin I<sup>b</sup> embryos complicate the analysis of these phenotypes. It will be interesting to determine if Mena<sup>geo</sup>/geo; profilin I<sup>b</sup> embryos that do not have obvious neural tube defects, but nonetheless die perinatally, suffer from other defects in cell migration or axon guidance. Interestingly, mutations in other actin-regulating proteins, such as the related Macmarcks and Marcks, are known to result in neural tube defects and exencephaly (Stumpo et al., 1995; Chen et al., 1996). Furthermore, deletion of both Arg and Abl, tyrosine kinases known to associate with actin (Van Etten et al., 1994), causes a collapse of the neural tube that is accompanied by the presence of ectopic actin-rich aggregates in the neuroepithelium (Koleske et al., 1998). Since Mena associates in vitro with the SH3 domains of Abl and Arg, and given the links between Abl signaling and Ena function in Drosophila, Koleske and colleagues speculate that the neurulation defect in Abl/Arg mutants may result from improper regulation of Mena.

Redundant and Unique Functions of Mena, EVL, and VASP

The phenotype of the Mena-deficient mice suggests that Mena may have a unique function in guidance of specific axons rather than, or perhaps in addition to, a general role in growth cone motility. This interpretation is supported by the finding that cultured embryonic hippocampal neurons from Mena-deficient mice appear, at least superficially, to develop normally (data not shown). It is therefore possible that, despite its localization at the tips of growth cone filopodia, Mena does not play a role in general growth cone motility. Alternatively, a role for Mena in general growth cone motility may be masked by the presence of the related family members EVL and VASP. Indeed, EVL and VASP are both expressed in portions of the developing nervous system and EVL is localized to the tips of growth cone filopodia (data not shown). The overlapping activities of Ena/VASP family members have been demonstrated by the ability of either Mena or VASP to rescue the viability of Drosophila Ena mutants (Ahem-Djamali et al., 1998; F. B. G. et al., unpublished data) and by experiments that show that Mena, EVL, and VASP are interchangeable in their ability to facilitate Listeria movement (F. B. G. et al., unpublished data).

Despite these redundancies, the phenotype of the Mena mutants suggests that Mena has a unique function not provided by EVL or VASP. This conclusion is supported by the fact that no CNS defects are detected in VASP-deficient mice (Aszodi et al., 1999). The unique function of Mena may be mediated by the neuronal specific 140 kDa Mena<sup>(+)</sup> isoform, which is known to have the ability to direct actin remodeling; EVL and VASP do not have neural specific variants and do not induce actin remodeling. The proposal that Mena<sup>(+)</sup> may play a role in commissural axon guidance is consistent with the finding that expression of Mena<sup>(+)</sup> peaks between E15 and P1, a time when the majority of the callosal and hippocampal commissure axons are migrating across the midline (Ozaki and Wahlsten, 1993).

Interestingly, Mena<sup>(+)</sup>, but neither the 80 kDa form of Mena, nor EVL or VASP, appears to be a substrate for tyrosine phosphorylation (Gertler et al., 1996; F. B. G. et al., unpublished data). In Drosophila, Ena is a target of Abl phosphorylation, and Ena function in axon guidance may be regulated by the opposing activities of Abl and the Dlar receptor tyrosine phosphatase (Wills et al., 1999a). How tyrosine phosphorylation affects Mena<sup>(+)</sup> function and whether Mena<sup>(+)</sup> is one of the phosphotyrosine substrates detected in the tips of growth cone filopodia remain to be determined. Nonetheless, it is possible that the Mena<sup>(-)</sup> isoform has a unique, tyrosine phosphorylation-dependent function in axon guidance analogous to that of Ena, while the 80 kDa form of Mena may have a more general function in growth cone and cell motility that can be partially replaced by EVL or VASP.

How Is Mena Localized in Growth Cone Filopodia?

The enrichment of Mena and/or Mena<sup>(+)</sup> at the distal tips of growth cone filopodia positions Mena appropriately to mediate axon guidance cues, but the mechanism by which Mena is enriched at the tips of filopodia remains unclear. In fibroblasts, Mena localization at focal adhesions is mediated by EVH1 domain interactions with the D/EFPPP-containing ligands Zyxin and Vinculin (Niebuhr et al., 1997). While Zyxin and Vinculin have been detected in filopodia of chicken sensory neurons (Gomez et al., 1996; Sydor et al., 1996), we have been unable to localize either molecule in filopodia of hippocampal neurons, suggesting that interaction with Vinculin and/or Zyxin is not the primary mechanism for
localization of Mena in these neurons. It therefore seems likely either that other EVH1 ligands exist or that localization in growth cones is mediated by ligands that interact with other portions of the Mena molecule.

Identification of one potential Mena-binding protein was made possible by the observation that the EVH1 domain of Mena, but not of EVL or VASP, binds with high affinity to peptides containing the sequence DLPPPP in which an L is substituted for the canonical F residue (Niebuhr et al., 1997). Based on this observation, Kidd and colleagues speculated that DLPPPP motifs present in the Robo/Sax3 family of axon guidance molecules may link this receptor to the actin cytoskeleton by recruiting Mena/Ena (Kidd et al., 1998). Robo is thought to repel axons from the midline, and, in Drosophila, loss of Robo results in promiscuous midline crossing (Kidd et al., 1998). In contrast, the Mena phenotype appears, based on anatomic analysis, to result from failure to cross the midline, while Ena mutants display only a mild Robo-like phenotype (Wills et al., 1999a). Therefore, the potential interaction between Robo and Ena/Mena is likely to represent only one aspect of the function of these molecules in axon guidance.

What Is the Function of Mena in Axon Guidance?
Growth cone motility requires the dynamic regulation of actin polymerization at the tips of filopodia. The fact that Mena(−/−) is localized at the tips of filopodia and can induce the protrusive activity of actin polymerization suggests that one function of Mena(+/+) may be to regulate actin polymerization in filopodia. In the absence of Mena, animals were sensitive to a 2-fold reduction in the levels of Profilin I, consistent with a model in which Mena and Profilin function in a common process. Unfortunately, the severe brain defects of the Menageo allele embryos prohibited analysis of axonal pathways in these animals. Nonetheless, it is tempting to speculate that Mena function in axon guidance involves interaction with Profilin. Biochemical data indicate that Profilin is a high-affinity ligand for Mena (Gettler et al., 1996) and that rapid movement of Listeria depends upon the ability of Ena/VASP proteins to recruit Profilin (Smith et al., 1996). Furthermore, Profilin itself has recently been shown to be essential for filopodial formation and neurite extension in cultured cells and for motor axon outgrowth in Drosophila (Suetsugu et al., 1998; Wills et al., 1999a) (this issue of Neuron).

It is possible that Mena influences cytoskeletal dynamics by physically concentrating Profilin in areas of the cell that require dynamic actin polymerization. In many cell types, however, Profilin is present in concentrations on the order of 100 μM and appears to be broadly distributed throughout the cell (Tseng et al., 1984), suggesting that physical concentration may not by itself be key to regulating Profilin function. While little is known about the concentration of Profilin in growth cones, similar reasoning may apply. Alternatively, the Mena-Profilin interaction may modulate Profilin function, thereby influencing actin dynamics.

The question of how Mena and Profilin ultimately regulate actin cytoskeletal dynamics is likely to be complex. A simple model based on studies of Listeria motility would predict that Ena/VASP family members function to recruit Profilin and enhance the rate of actin polymerization and cell motility (Pollard, 1995). Based on this model, one might predict that mutations in either Ena/VASP proteins or in Profilin would lead to decreased cell motility. Genetic studies indicate, however, that loss of Ena/VASP family members can actually enhance cell motility. In mice, loss of VASP increases the rate of platelet aggregation and attenuates the cyclic nucleotide-mediated inhibition of aggregation (Aszodi et al., 1999). If the rate of platelet aggregation is directly related to the rate of actin polymerization, then the simplest interpretation of these data is that VASP normally retards the rate of actin assembly and that this inhibitory function is potentiated by PKA phosphorylation. In Drosophila, loss of Ena leads to a highly penetrant “bypass” phenotype in which growth cones fail to stop or turn at the appropriate choice points, while mutations in Profilin lead to a “stop short” phenotype in which growth cones fail to advance beyond the choice point (Wills et al., 1999a, 1999b). Thus, although both Ena and Profilin appear to have important functions at choice points, their role in regulating growth cone dynamics is likely to involve more than simple changes in the rate of actin polymerization.

A recent report indicates that Mena and its homologs may be directly involved in response to the Netrin signaling pathway. Unc34, the C. elegans homolog of Mena, was identified in a screen for suppressors of axonal guidance defects induced by ectopic expression of the Netrin receptor Unc5 (Colavita and Culotti, 1998). In vertebrates, Netrin-1 and DCC, a Netrin receptor that mediates chemoattractive responses, are required for spinal commissural axon guidance and formation of the corpus callosum and the hippocampal and anterior commissures (Serafini et al., 1996; Fazeli et al., 1997). Although the cytoplasmic targets of the Netrin receptors have not been identified, it is known that Netrin-1 signaling is modulated by cyclic nucleotide-dependent protein kinases (Ming et al., 1997). This ability of second messenger signaling to modify growth cone chemotaxis may be one way in which axons can integrate signals from multiple guidance systems. Given that fact that Mena is an in vivo substrate for PKA, as well as the phenotype of the Mena mutants and the genetic interaction between Unc34/Mena and the Netrin signaling pathway, it seems possible that Mena and its relatives may represent a convergence point for these signaling pathways. Experiments are underway to determine if Mena is one of the critical factors that transduce these diverse signals into the changes in cytoskeletal dynamics required for growth cone guidance.

Experimental Procedures

Targeted Disruption of Mena
A targeting vector was constructed by fusing an 8 kb fragment of genomic DNA from the first intron of Mena to a splice-acceptorigeo cassette with a polyadenylation site (Friedrich and Soriano, 1991) followed by a 1.1 kb fragment from the third intron of Mena. A PGK-diphtheria toxin cassette was inserted after the short arm fragment for negative selection. Further details of the construction and the Mena genomic locus are available upon request. The targeting vector was electroporated into AK7 ES cells (Imamoto and
Soriano, 1993). Correctly targeted clones were identified by PCR and verified by Southern blotting analysis. Four ES clones were used to generate chimeras by blastocyst injection. Germ-line transmission of the disrupted allele was verified by PCR and Southern blot analysis. ES cell culture and blastocyst injection were performed as described (Friedrich and Soriano, 1991).

Western Blot Analysis
Organs were dissected and placed in buffer (RIPA; 50 mM Tris [pH 8.0], 150 mM NaCl, 1% Triton, 0.5% deoxycholate, 0.1% SDS, 1 mM pepstatin, 1 mM PMSF, 0.3 mM aprotinin, 1 mM Leupeptin, 5 mM E-64, 1 mM EDTA, 1 mM sodium vanadate, 50 mM sodium fluoride, 2 mM levamisole, 30 mM sodium pyrophosphate), the tissues were dissociated using a tissue homogenizer, and extracts were centrifuged for 30 min at 100,000 g. Protein concentrations were determined using the BCA assay (Pierce). SDS-PAGE electrophoresis and Western blotting were done using standard techniques. Antibodies included anti-Mena (polyclonal antiserum 2197), anti-Tubulin (polyclonal T3526, Sigma), anti-EVL (monoclonal 84H11), anti-VASP (polyclonal M4, Alexis Corp.), and horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse (Jackson ImmunoResearch). Signal was developed using the Renaissance chemiluminescence reagent (DuPont-NEN).

In Situ Hybridization
In situ hybridization was done following protocol 2 as described (Hogan et al., 1994). Probes were prepared by in vitro transcription of linearized template DNA (either the entire Mena coding sequence or the [+] exon alone) using digoxigenin labeled nucleotides (Boehringer Mannheim). Sense and antisense probes were prepared using the T7 and T3 promoters at opposite ends of the linearized template. Signal was developed using an alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) and BCIP/NBT (Vector Labs).

Tissue Preparation and Histological Analysis
Animals were anesthetized with Avertin and transcardially perfused with 4% PFA (for silver staining, DIL labeling, and Immunocytochemistry) or in 30% sucrose followed by 4% PFA (X-gal histochemistry and subsequent anti-Py, anti-MAP-2, or anti-neurofilament Immunocytochemistry; Snyder et al., 1997). Silver staining was done as described (Pink and Heimer, 1967). X-gal histochemistry was developed as described (Hogan et al., 1994). Specimens were either embedded in OCT and cut on a cryostat (Figures 4A and 4B) or were frozen and cut using a freezing microtome (Figures 4C–4H). After X-gal histochemistry, sections were rinsed, blocked, and incubated sequentially with primary antibodies (anti-Py [Woodhams et al., 1989] or anti-neurofilament [Amersham]), biotinylated secondary antibody, avidin-biotin complex (Vector Laboratories), and diamobenzidine substrate (Pierce).

Dil Labeling
After perfusion, P0 brain specimens were placed in fresh 4% PFA for 2–3 days. A glass capillary (outside diameter 70 μm), attached to a Nanojector (Drummond Scientific Co.), was filled with 0.3 μl of 10% Dil in 100% ETOH (Honig and Hume, 1989) and lowered 0.5 μm deep into the cortical surface at five sites along the rostral-caudal midline (i.e., cingulate cortex), and 50 nl of the Dil was slowly injected. The specimen was subsequently placed back in fresh 4% PFA and incubated at 37°C for 8–10 weeks, after which 50 μm coronal sections were cut using a vibrating microtome (Vibratome) and mounted with Fluromount G (Electron Microscopy Sciences).

Cell Culture and Immunocytochemistry
Primary hippocampal neurons were prepared from E16 mouse as described for E18 rat (Goslin and Banker, 1991). After 24 hrs, cells were fixed in 4% PFA/PBS, blocked with 10% BSA/PBS, and permeabilized with 0.2% Triton-X100/PBS. Primary antibodies included poly- and monoclonal anti-Mena antibodies, polyclonal anti-(+) exon antibody, and monoclonal antibody 13H9 (Goslin et al., 1989). Secondary antibodies included cy-5-goat anti-rabbit, Texas-red donkey anti-rabbit, and FITC-donkey anti-mouse (Jackson ImmunoResearch). Coverslips were mounted with DABCO in polyvinyl alcohol and imaged using a DELTAVISION deconvolution imaging microscope.

Glia cultures were prepared from P0 mouse cortex and plated onto tissue culture dishes in plating medium (MEM, 10% horse serum, 0.6% glucose). Cortical cultures were prepared from E15 mice, plated on poly-L-lysine coated dishes and maintained in serum-free medium (LIJ F-12/DMEM supplemented as described for serum-free hippocampal culture, except using 25 μg/ml insulin). Under these conditions, the majority of the cells are neuronal, though non-dividing glia persist.

Analysis of Mena-profilin Animals
The construction of the profun I targeting vector will be reported elsewhere (W. W. and D. K., unpublished data). The profin I mutation was generated in J1 ES cells, which were derived from a 129sv strain (Li et al., 1992). profun I heterozygotes were bred with Mena-supero heterozygotes. The profin I, Mena-supero double heterozygous progeny were intercrossed for the analysis presented. Consistent results from this cross were observed with these animals and with double heterozygous animals that had been backcrossed into a congenic 129sv strain for up to four generations. Although it is formally possible that the defects seen in the Mena-supero/profun I animals are associated with a tightly linked locus, it is striking that we analyzed over a hundred animals and never observed neural tube defects segregating independently of the profun I allele. E9.5 embryos from Mena-profilin crosses were dissected, tails were removed for genotyping (see above), and embryos were fixed in 4% PFA, then photographed, and stored in PBS. Embryos for scanning electron microscopy were dehydrated in methanol/PBST, washed in methanol, and allowed to air dry for 5 min prior to mounting with adhesive tape.

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