Negative Regulation of Fibroblast Motility by Ena/VASP Proteins

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Summary

Ena/VASP proteins have been implicated in cell motility through regulation of the actin cytoskeleton and are found at focal adhesions and the leading edge. Using overexpression, loss-of-function, and inhibitory approaches, we find that Ena/VASP proteins negatively regulate fibroblast motility. A dose-dependent decrease in movement is observed when Ena/VASP proteins are overexpressed in fibroblasts. Neutralization or deletion of all Ena/VASP proteins results in increased cell movement. Selective depletion of Ena/VASP proteins from focal adhesions, but not the leading edge, has no effect on motility. Constitutive membrane targeting of Ena/VASP proteins inhibits motility. These results are in marked contrast to current models for Ena/VASP function derived mainly from their role in the actin-driven movement of *Listeria monocytogenes*.

Introduction

How a cell moves remains a compelling mystery. Cell migration is required for processes such as immune cell homing, wound healing, and axonal pathfinding. Migration requires the coordinated execution and integration of complex individual processes. In its simplest form, movement requires that a cell generate and maintain a state of asymmetry or polarity.

Once polarized, a cell must execute a four-step cycle to translocate (Lauffenburger and Horwitz, 1996). First, a cell must extend a leading edge in the direction of movement. Increased actin polymerization at the leading edge arises from creation of new barbed ends oriented toward the membrane, either by nucleation of new filaments from pools of G actin or by severing or uncapping existing filaments. Actin monomers are added onto barbed ends until they are capped (Schafer and Cooper, 1995). Actin nucleation and filament elongation are thought to play a critical role in leading edge protrusion (Lauffenburger and Horwitz, 1996). Second,

once a cell has extended a process, it must form semi-stable attachments to the substratum. One class of attachment points, focal adhesions, contains aggregates of integrins and a variety of cytosolic signaling and cytoskeletal proteins, and serve as sites of bidirectional signaling between the extracellular matrix and the actin cytoskeleton (Schoenwaelder and Burridge, 1999). Third, once a cell has extended and anchored a new process, it must slide the cell body forward by traction. The fourth step is release of substratum attachment points at the rear of the cell.

The Ena/VASP protein family has been implicated in the regulation of cell migration (Gertler et al., 1996). Enabled (Ena) was identified as a genetic suppressor of mutations in *Drosophila* Ableson tyrosine kinase (D-Abl) (Gertler et al., 1990). VASP was identified as an abundant substrate for cyclic-nucleotide dependent kinases in platelets (Halbrugge and Walter, 1990). Two other mammalian family members, Mena (*m*ammalian *Ena*bled) and EVL (*E*na-*V*ASP *l*ike), were identified by sequence similarity (Gertler et al., 1996).

Ena/VASP family members share a conserved domain structure. The N-terminal third of the protein, the EVH1 (*E*na *V*ASP *h*omology) domain (Gertler et al., 1996), mediates subcellular targeting to focal adhesions by binding to proteins containing a motif whose consensus is D/E FPPPPXD (Niebuhr et al., 1997). The phenylalanine residue of the motif, along with flanking acidic residues, are critical for optimal binding (Carl et al., 1999). The EVH1 ligand motif is found in several proteins, including the focal adhesion proteins zyxin and vinculin. The central portion of Ena/VASP proteins contains proline-rich stretches that bind to three types of proteins: profilin, SH3, and WW domain-containing proteins (Gertler et al., 1996; Ermekova et al., 1997). The C-terminal third of Ena/VASP proteins contains the EVH2 domain that binds in vitro to F actin and has a putative coiled-coil region thought to be important for multimerization (Bachmann et al., 1999).

The localization of Ena/VASP proteins suggests they may be involved in regulating actin dynamics and/or adhesion. In fibroblasts, Ena/VASP proteins localize to focal adhesions and to the leading edge, while in neuronal growth cones, they are concentrated at the distal tips of filopodia (Reinhard et al., 1992; Gertler et al., 1996; Lanier et al., 1999). Genetic analyses of Ena/VASP family members in flies and mice demonstrated that these proteins function in processes that involve cell shape change and movement, including platelet aggregation and axon guidance (Aszodi et al., 1999; Lanier et al., 1999; Wills et al., 1999). In mice, a dosage-sensitive genetic interaction between Mena and profilin I supports a model in which these proteins function in concert during development.

Ena/VASP proteins are also implicated in actin dynamics by their role in facilitating the actin-based motility of the intracellular bacterial pathogen *Listeria monocytogenes* (Chakraborty et al., 1995). The *Listeria* protein ActA is required for formation of actin tails characteristic

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of motile bacteria (Domann et al., 1992). ActA is a multi-domain protein on the bacterial surface that interacts with host cell factors to trigger actin assembly (Pistor et al., 1995). Actin nucleation is driven by ActA-mediated activation of the Arp2/3 complex (Welch et al., 1998). Ena/VASP proteins are the only host cell factors known to bind directly to ActA, which contains four copies of the EVH1 ligand motif (D/E FPPPPXD) (Niebuhr et al., 1997). Mutation of these repeats leads to a defect in bacterial movement, although an actin cloud and short actin tails still form around the bacterium (Smith et al., 1996; Niebuhr et al., 1997). In vitro experiments using depleted, cell-free extracts and reconstitution with purified components demonstrated that Ena/VASP proteins are required for efficient actin tail formation and normal bacterial motility (Laurent et al., 1999; Loisel et al., 1999).

Listeria movement has been proposed as a model for actin reorganization at the leading edge of motile cells. Recent work using GFP-tagged VASP demonstrated a strong correlation between membrane extension rates and concentration of VASP at the leading edge (Rottner et al., 1999). Based on localization studies and Listeria experiments, it has been proposed that Ena/VASP proteins promote actin-based cell movement. We tested this hypothesis directly by overexpressing or neutralizing Ena/VASP proteins in motile fibroblasts and by examining cells lacking all Ena/VASP proteins. Surprisingly, we report that Ena/VASP proteins function as negative regulators of fibroblast motility and lamellipodial extension.

Results

Overexpression of Mena Inhibits Cell Motility in a Dose-Dependent Manner

To test the hypothesis that Ena/VASP proteins promote cell motility, we first analyzed the effect of elevated Mena or VASP levels on cell migration. Rat2 fibroblasts were infected with a retrovirus that drives expression of an EGFP (enhanced green fluorescent protein)-Mena fusion protein. In these cells, GFP signal distribution corresponded to that of endogenous Mena in uninfected cells (Figure 1A and Video S1 [see Supplemental Data below]). In addition, F actin distribution was not visibly altered by overexpression of the fusion protein. Since EGFP-Mena was expressed from a stably integrated provirus, cells could be sorted by FACS into populations containing increasing amounts of fusion protein as detected by GFP signal. Cell lysates from populations sorted for low, medium, or high levels of GFP were prepared and analyzed by quantitative Western blotting using anti-Mena antisera. The low, medium, and high overexpressing populations contained a total of 1.9-, 2.7-, and 3.9-fold more Mena, respectively, than uninfected Rat2 controls (Figure 1B).

To address the effect of EGFP-Mena overexpression on cell motility, cells were analyzed by time-lapse videomicroscopy. Movies of migrating cells were digitized to allow tracing of cell outlines and calculation of centroid positions. Cell speed was calculated from the paths of cells. Cells were tested within two passages of sorting to reduce possible phenotypic drift associated with compensation mechanisms such as up- or downregula-
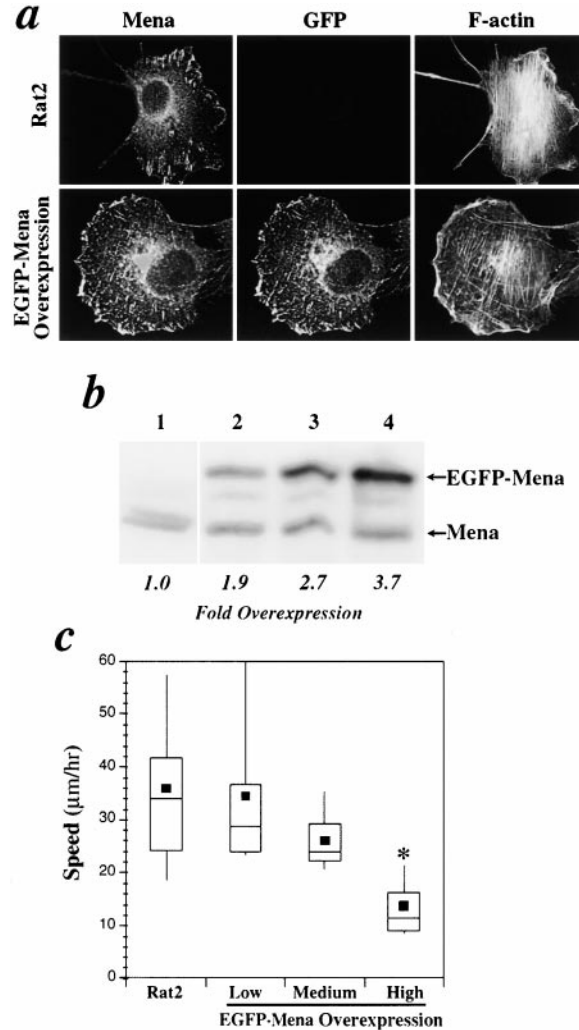

Figure 1. Overexpression of EGFP-Mena Inhibits Cell Motility in a Dose-Responsive Manner

(A) Immunofluorescence of uninfected Rat2 fibroblasts and Rat2 cells expressing EGFP-Mena fusion protein (medium population).
(B) Western blot of control uninfected cells (1) and those sorted for increasing GFP signal (2–4) probed with anti-Mena polyclonal antibody.
(C) Box and whisker plots of speed. Dot indicates mean, middle line of box indicates median, top of box indicates 75th quatrile, bottom of box indicates 25th quatrile, and "whiskers" indicate extent of 10th and 90th percentiles, respectively. At least 20 cells per treatment from 2–3 separate experiments were analyzed by one-way ANOVA. p value was <0.0001 and treatments with nonoverlapping 95% confidence intervals are marked by an asterisk.

tion of other adhesion or motility genes. Surprisingly, cell speeds decreased with increasing levels of EGFP-Mena expression (Figures 1C and 3A and Video S2). In addition, cells expressing the highest levels of EGFP-Mena showed a distinct lack of polarity (data not shown). Similar results were obtained by overexpressing an untagged VASP protein (data not shown), indicating that VASP overexpression has a similar effect on motility and that the EGFP-Mena phenotype is not simply due to the EGFP tag. Therefore, increased levels of Mena or VASP retard fibroblast motility.
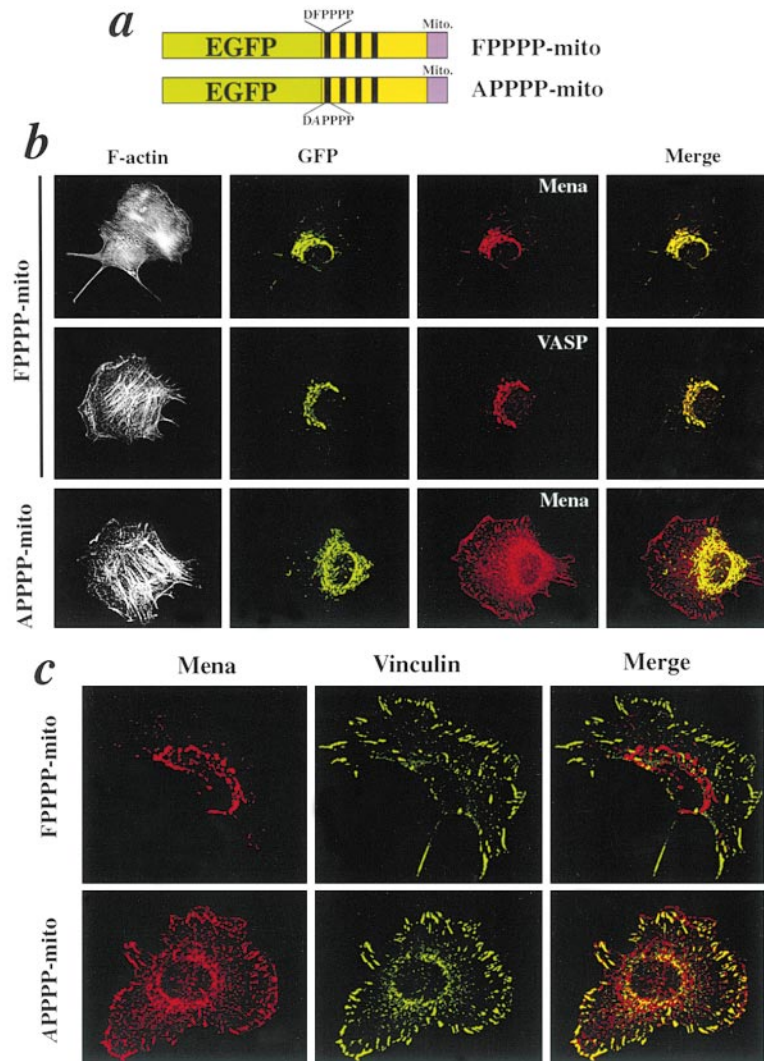
Figure 2. Expression of a Mitochondria-Targeted Ena/VASP-Binding Protein Sequesters All Mena and VASP, but Leaves Other Focal Adhesion Proteins in Place

(A) Schematic diagram of mito targeting constructs. (B) Immunofluorescence of FPPPP-mito and APPPP-mito expressing Rat2 cells. Images were collected in monochrome and pseudocolored to show degree of overlap between GFP and Mena. (C) Mena and vinculin overlap in FPPPP- and APPPP-mito cells.

Sequestration of Ena/VASP Proteins on the Mitochondria Increases Cell Speed

The role of Ena/VASP proteins in motility most likely depends upon their presence in either focal adhesions, the leading edge, or both. We reasoned that depletion of Ena/VASP proteins from these structures should inhibit their function. We exploited the ability of the EVH1 domain to target Ena/VASP proteins within cells to sequester all Ena/VASP proteins on the mitochondrial surface. When expressed in eukaryotic cells, ActA is targeted to the outer mitochondrial membrane. This observation was used to map the binding site for VASP and actin-nucleation domains within ActA (Pistor et al., 1994, 1995). We generated a construct that directs a fusion between EGFP and the four EVH1-binding sites (D/EFPPPPXD) of ActA to the mitochondrial surface ("FPPPP-mito", Figure 2A). This construct lacks all N-terminal residues of ActA, including those shown to activate the Arp2/3 complex and drive actin nucleation, and simply places EVH1 binding sites on the mitochondrial surface. A parallel construct was made in which the phenylalanine residue essential for EVH1-binding was changed to an alanine in each of the four FPPPP repeats. This construct, "APPPP-mito", does not bind Ena/VASP proteins, but contains all other sequences present in the original construct and serves as a specificity control for Ena/VASP-independent effects. Rat2 cells were infected with retroviruses that express these fusion proteins and sorted by FACS into populations of cells that were >98% GFP$^+$ and expressed equivalent levels of the FPPPP-mito or APPPP-mito constructs (data not shown).

The distribution of Ena/VASP proteins in the FPPPP-mito expressing cells was analyzed by immunofluorescence. Instead of normal focal adhesion and membrane localization, all detectable Mena or VASP signals were colocalized with GFP on mitochondria (Figure 2B). As expected, APPPP-mito cells showed GFP-labeled mitochondria, but the Mena signal was distributed normally in focal adhesions and the leading edge with little or no overlap with mitochondria. VASP also showed no colocalization with mitochondria in APPPP-mito cells (data not shown). EVL expression is not detectable by immunofluorescence in Rat2 cells, so anti-EVL staining was not performed on FPPPP-mito cells. Interestingly,

a diffuse cytoplasmic signal, previously thought to be background signal, was not detected for Mena in the FPPPP-mito cells (Figure 2B). This suggests that a pool of Mena protein exists in the cytosol that is not associated with focal adhesions or the leading edge under normal circumstances. Together, these results indicate that the FPPPP-mito construct effectively depletes all detectable Mena and VASP protein from the leading edge, focal adhesions, and cytosol and sequesters it on the mitochondrial surface.

The FPPPP-mito expressing cells were stained with phalloidin to determine if mitochondrial sequestration of Ena/VASP proteins caused detectable changes in F actin distribution. All normal forms of F actin for fibroblasts (stress fibers, ruffles, and microspikes) were present in FPPPP-mito cells and indistinguishable from APPPP-mito and parental control cells. Furthermore, no unusual enrichment of F actin was observed on or near mitochondria, indicating that clustering of endogenous Ena/VASP proteins in vivo is not sufficient to recruit or assemble detectable levels of F actin.

FPPPP-mito, EGFP-Mena overexpressing ("high" population), and control cells were each stained after complete spreading with a battery of focal adhesion markers (FAK, vinculin, paxillin, tensin, phosphotyrosine, and zyxin). When the distributions of Mena and vinculin were examined in FPPPP-mito cells, vinculin and the other markers remained in their normal location at focal adhesions and were not recruited to the mitochondria along with Mena (Figure 2C and Supplemental Table 1). Observation of staining patterns revealed no qualitative differences in distribution or appearance of focal adhesions in any of the cell populations (data not shown). When numbers of vinculin- or zyxin-positive focal adhesions were counted, there were no significant differences between the various cell populations (data not shown). During spreading, all cell populations contained equivalent numbers of zyxin-positive focal adhesions at 15 and 45 min (data not shown).

The FPPPP-mito cells were analyzed by videomicroscopy. Sequestration of all Ena/VASP protein on mitochondria caused cells to move significantly faster than either uninfected controls or APPPP-mito cells (Figures 3A and 3B and Video S3). These cells showed no detectable upregulation of Mena or VASP by Western blot even after five or more passages and appeared to grow and divide indistinguishably from controls (data not shown), indicating that Ena/VASP proteins are dispensable for cytokinesis. These results indicate that Mena and VASP are not required in focal adhesions or at the leading edge for cell movement, and that speed actually increases when Mena and VASP are removed from their normal locations.

Complementation of Ena/VASP-Deficient Cells with Mena Reduces Cell Speeds

The experiments using FPPPP-mito depend upon the EVH1-ligand to recruit specifically all endogenous Ena/VASP proteins. While control experiments using APPPP-mito demonstrate that the observed effects on cell speed correlate with redistribution of Ena/VASP proteins, it is possible that other ligands exist that bind
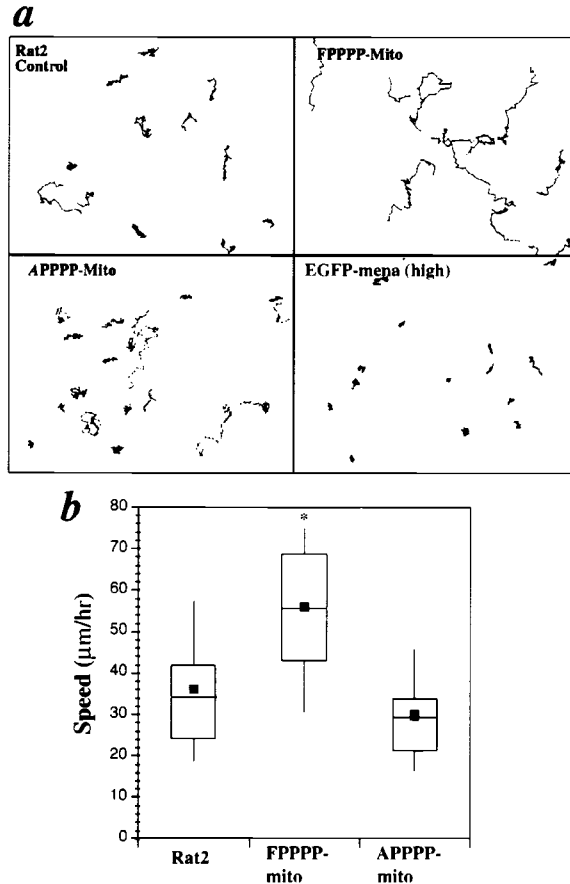

Figure 3. Sequestration of Ena/VASP Proteins Stimulates Cell Motility

(A) Cell paths during a 4.5 hr migration experiment. Dots show centroid positions at 5 min intervals. (B) Box and whisker plots of cell speeds. Data were analyzed as in Figure 1C (ANOVA p value < 0.0001).

selectively to the FPPPP motif and contribute to the observed phenotypes. Formal proof that Ena/VASP proteins negatively regulate cell speed required analysis of cells lacking all Ena/VASP proteins.

To isolate Ena/VASP protein-deficient cells, we cultured fibroblast populations from Mena/VASP (MV) double homozygous mutant embryos. Phenotypic analysis of embryonic defects observed in MV double mutants is in progress and will be reported elsewhere. Many cell types express two or all three Ena/VASP family members; therefore, clonal derivatives of MV fibroblast populations were stained with an antibody to identify cell lines lacking detectable expression of EVL, the remaining family member. A representative line, MV[D7], was selected for further characterization, and lack of detectable levels of Mena, VASP, and EVL was confirmed by Western blot analysis (Figure 4A).

If the increased speeds observed in cells expressing the FPPPP-mito construct are due to specific effects on Ena/VASP proteins, then cells lacking Ena/VASP proteins should be refractory to expression of FPPPP-mito. To test this hypothesis, populations of MV[D7] expressing FPPPP-mito were generated and analyzed by time-lapse videomicroscopy. The speeds of MV[D7]/FPPPP-
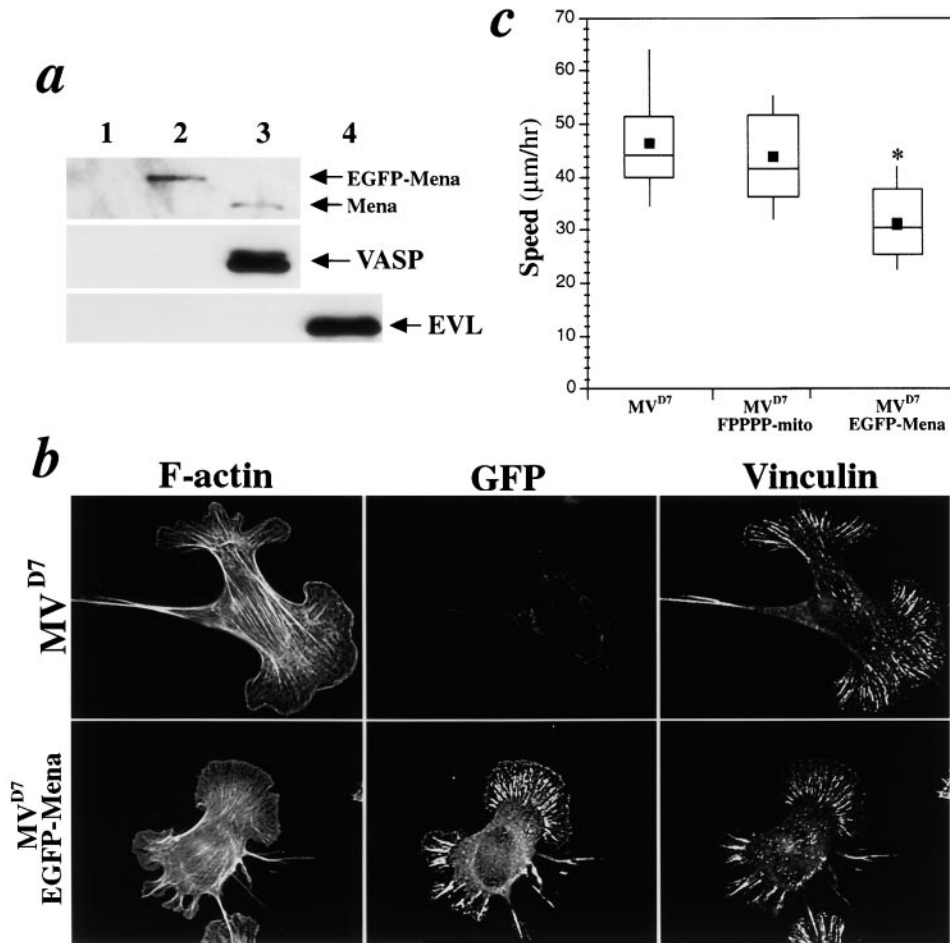
Figure 4. Complementation of Ena/VASP-Deficient Cells Slows Motility

(A) Western blot of MV^{D7} (1), MV^{D7}/EGFP-Mena (2), Rat2 (3) and Rat2 cells transfected with EVL (4) RIPA extracts with antibodies to each Ena/VASP family member. (B) Immunofluorescence of MV^{D7} and MV^{D7}/EGFP-Mena cells. (C) Box and whisker plots of cell speed (ANOVA p value < 0.0001).

mito cells were statistically indistinguishable from those of parental MV^{D7} cells (Figure 4C). This result strongly suggests that the phenotype induced by expression of FPPPP-mito results from a specific perturbation of Ena/VASP proteins.

If absence of Ena/VASP proteins results in increased cell speeds, then rescue of MV^{D7} cells by expression of an Ena/VASP protein should reduce speeds. MV^{D7} cells were infected with the EGFP-Mena retrovirus and sorted by FACS to create a population of MV^{D7} cells that express moderate levels of Mena (MV^{D7}/EGFP-Mena). This cell line is identical in every way to the MV^{D7} line except for Mena expression. Complementation of the clonal MV^{D7} line was chosen over use of control cells from wild-type littermate embryos to circumvent the difficulty of isolating an isotypic cell line. FACS analysis indicated that, on an average per cell basis, MV^{D7}/EGFP-Mena cells express a level of EGFP-Mena roughly equivalent to the "low" population of EGFP-Mena overexpressing Rat2 cells in Figure 1 (data not shown). Since the amount of EGFP-Mena in the low population is similar to the amount of endogenous Mena in Rat2 cells, the MV^{D7}/EGFP-Mena cells express Mena at a level roughly comparable to Rat2 fibroblasts. The MV^{D7} and MV^{D7}/EGFP-

Mena cell lines were stained with probes to vinculin and F actin and examined to verify proper distribution of EGFP-Mena (Figure 4B). No gross differences were observed between the two cell lines, indicating that deficiency of all Ena/VASP proteins has no effect on the appearance of focal adhesions or distribution of F actin.

The migration rates of MV^{D7} and MV^{D7}/EGFP-Mena cells were analyzed by videomicroscopy. MV^{D7} cells migrated significantly faster than MV^{D7}/EGFP-Mena cells (Figure 4C), indicating that cell speeds are reduced by complementation of Ena/VASP-deficient cells with Mena. When combined with the data from the Rat2 cell populations, these results provide compelling evidence that cell motility rates are increased in the absence of Ena/VASP proteins.

Displacement of Ena/VASP Proteins from Focal Adhesions, but Not the Leading Edge, Has No Effect on Cell Motility

We tested the requirements for Ena/VASP proteins in focal adhesions using a modified construct containing EGFP and the FPPPP repeats but lacking the mitochon-
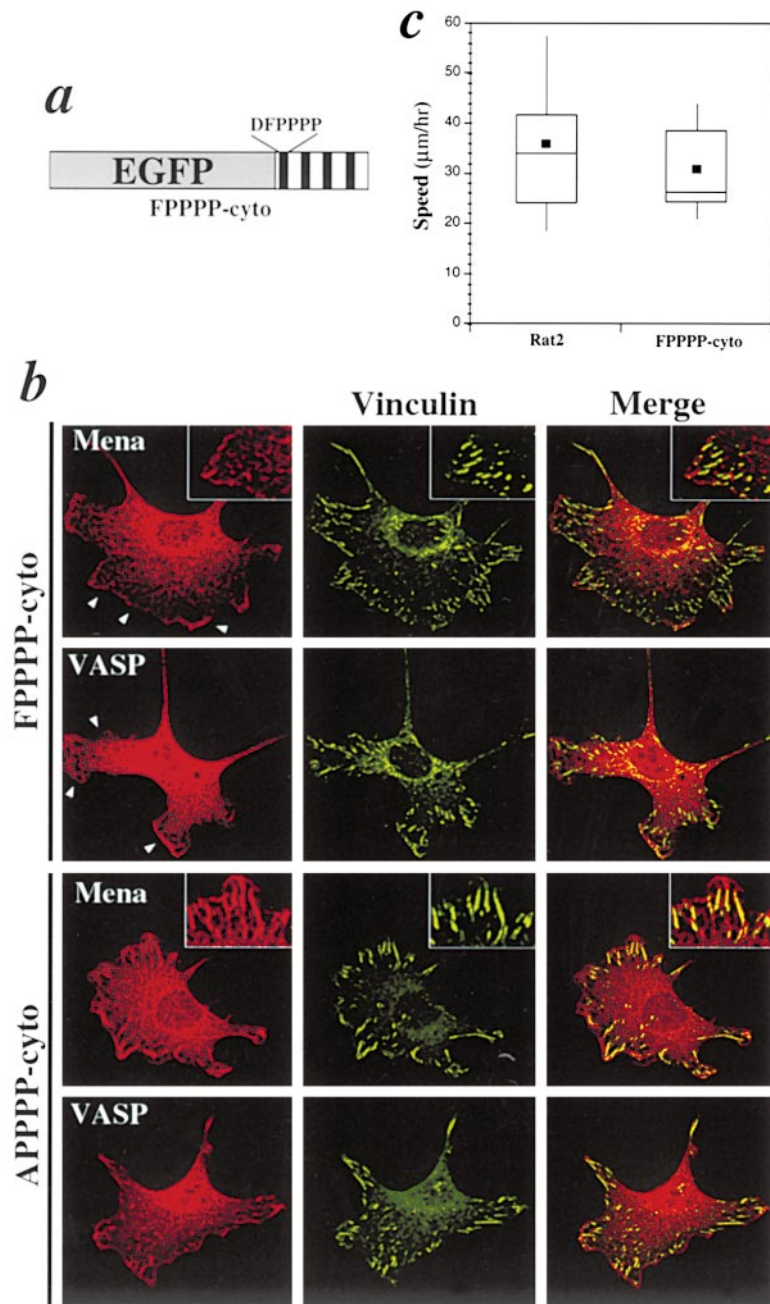
Figure 5. Depletion of Ena/VASP Proteins from Focal Adhesions, but Not the Leading Edge, Has No Effect on Cell Motility

(A) Schematic diagram of cytoplasmic construct. (B) Immunofluorescence of FPPPP-cyto and APPPP-cyto cells stained for vinculin and either Mena or VASP. Note leading edge and ruffle staining (arrowheads) of Mena and VASP in FPPPP-cyto cells and lack of overlap at vinculin positive focal adhesions. Insets show 2× magnification. (C) Box and whisker plots of cell speeds (p value from Student's t test was >0.05).

drial targeting sequence (Figure 5A). This construct, FPPPP-cyto, localizes to the nucleus and cytoplasm and effectively displaced Mena and VASP from focal adhesions (Figure 5B). Interestingly, FPPPP-cyto cells retained distinct Mena and VASP staining at ruffles and the leading edge (Figure 5B, arrowheads). As with FPPPP-mito cells, F-actin and vinculin staining remained unchanged in FPPPP-cyto cells (data not shown and Figure 5B). Cells expressing the negative control version of this construct, APPPP-cyto, showed Mena and VASP staining indistinguishable from controls (Figure 5B). These results indicate that while recruitment of Ena/VASP proteins to focal adhesions requires interac-

tions with FPPPP-containing proteins, targeting to the leading edge can occur by other mechanisms.

To test for effects on cell motility, populations of Rat2 cells expressing FPPPP-cyto or APPPP-cyto control constructs were analyzed by videomicroscopy. The FPPPP-cyto construct caused no change in cell speeds (Figure 5C). The motility properties of cells expressing APPPP-cyto also did not differ significantly from parental cells (data not shown). These results indicate that displacement of Mena and VASP from focal adhesions has no effect on cell motility under the assay conditions. Therefore, the increased cell speeds observed in FPPPP-mito cells likely result from depletion of Mena
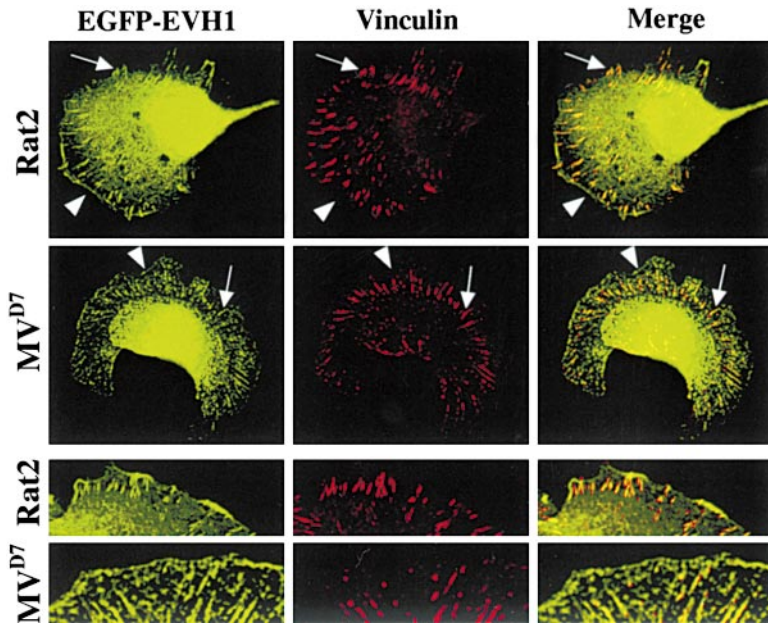
Figure 6. Leading Edge as Well as Focal Adhesion Targeting of Mena Is Mediated by the EVH1 Domain

Immunofluorescence analysis of EGFP-EVH1 expressing Rat2 and MV^{D7} cells. Arrowheads and arrows indicate leading edge membrane and focal adhesions, respectively. Bottom panels show close-ups of different cells.

and VASP from either the cytosol or leading edge, but not from focal adhesions.

The EVH1 Domain Is Sufficient to Mediate Leading Edge Targeting of Ena/VASP Proteins

Since recruitment of Ena/VASP proteins to the leading edge can occur independently of interactions between the EVH1 domain and FPPPP-containing proteins, we wondered if the EVH1 domain could mediate leading edge localization. We expressed a fusion of the Mena EVH1 domain with EGFP in Rat2 cells or MV^{D7} cells and found that the EVH1 domain directed GFP to both focal adhesions and the leading edge (Figure 6, arrows and arrowheads). Focal adhesion and leading edge EGFP-EVH1 signal is not as robust as with full-length EGFP-Mena, perhaps because these molecules cannot multimerize through the EVH2 domain to increase signal strength. In Rat2 cells, this construct may also be in competition for limited binding sites with more avid, multimerized endogenous Ena/VASP complexes. EGFP-EVH1, unlike full-length EGFP-Mena, is detected in the nucleus, perhaps because it lacks a nuclear exclusion signal or is simply small enough to diffuse into the nucleus. These results indicate that, in fibroblasts, the EVH1 domain is sufficient to direct leading edge localization as well as focal adhesion targeting.

Redistribution of Ena/VASP Proteins to the Plasma Membrane Decreases Cell Motility

Together, the results of the FPPPP-mito and FPPPP-cyto experiments indicated that either the cytosolic or leading edge pools of Ena/VASP proteins were likely responsible for the changes in motility observed. To distinguish between these possibilities, the FPPPP-cyto construct was modified to include the lipid modification domain (CAAX box) from H-ras. This construct directs EVH1 binding sites to the inner leaflet of the plasma

membrane. As before, two constructs were prepared containing either intact (FPPPP-CAAX) or mutant (APPPP-CAAX) repeats (Figure 7A). Rat2 cells infected with FPPPP-CAAX and APPPP-CAAX retroviruses were sorted by FACS for equivalent GFP expression. When these cells were examined for Mena and GFP localization by immunocytochemistry, all the Mena was redistributed to the plasma membrane in FPPPP-CAAX cells, but not in those expressing APPPP-CAAX (Figure 7B). F-actin distribution remained largely unchanged in FPPPP-CAAX cells, although these cells showed some increased propensity to form ruffles. When these cells were analyzed by videomicroscopy, FPPPP-CAAX cells exhibited almost no ability to translocate (Figure 7C), while the speed of APPPP-CAAX cells was statistically indistinguishable from uninfected control cells (Figure 7C). Although FPPPP-CAAX cells could attach and spread with apparently normal kinetics, they very rarely adopted the polarized morphology characteristic of a motile cell (data not shown). These results indicate that Ena/VASP proteins inhibit motility when directed to the plasma membrane.

Ena/VASP Proteins Act to Inhibit the Rate of Membrane Extension and Retraction

The dramatic changes in cell motility observed by changing the level or subcellular distribution of Mena and VASP suggested that these proteins may play a role in regulating membrane extension. To investigate this, movies of the various cell populations were compared for the parameter of cell shape change. To measure shape change, the outlines of cells in adjacent movie frames were compared. Newly protruded area (membrane extension) is termed positive flow, while membrane area retracted from the previous frame is negative flow (Figure 8A, green and red, respectively). When averaged over the course of the movie, positive flow nearly always matches negative flow since Rat2 cells do not
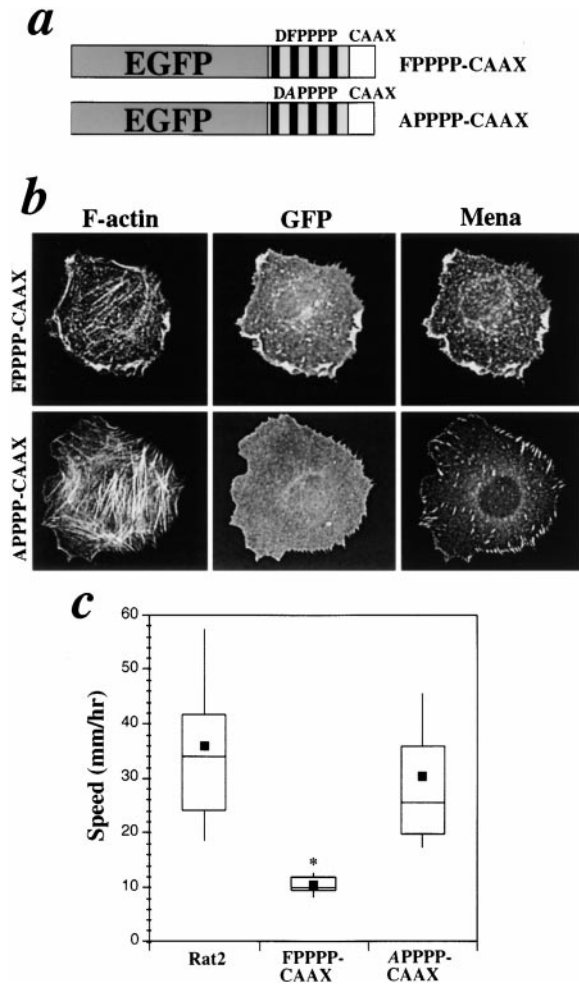
Figure 7. Constitutive Targeting of Ena/VASP Proteins to the Plasma Membrane Inhibits Cell Motility

(A) Schematic diagram of membrane targeting constructs. (B) Immunofluorescence of FPPPP-CAAX and APPPP-CAAX expressing cells. (C) Box and whisker plots of cell speed (ANOVA p value < 0.0001).
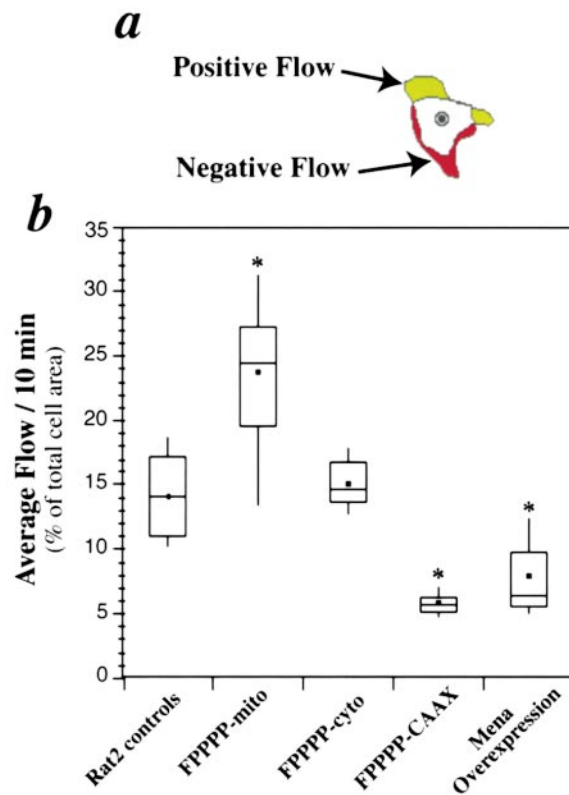

Figure 8. Protrusion and Retraction, Independent of Cell Translocation, Positively Correlates with Speed

(A) Diagram illustrating positive and negative membrane flow. The outlines of the same cell in two adjacent frames are overlaid; new areas of protrusion are indicated in green and areas of retraction are in red. (B) Box and whisker plot of average flow per 10 min time period. Average flow is calculated by averaging the absolute values of positive and negative flow and is expressed as a percentage of total cell area from the first frame (ANOVA p value < 0.0001).

Discussion

Ena/VASP Proteins Act as Negative Regulators of Fibroblast Motility

The major conclusion of this work is that Ena/VASP proteins *negatively* regulate fibroblast movement and membrane extension. These experiments were undertaken with the expectation that Ena/VASP proteins would promote cell motility, but our results do not support this hypothesis. Overexpression of Mena or VASP caused a dose-dependent decrease in cell speed. Constitutive membrane targeting of endogenous Mena and VASP also decreased cell speed. In contrast, sequestration of all Ena/VASP proteins on mitochondria resulted in a striking increase in cell speed. The ability to generate such reciprocal effects via increase or decrease of particular pools of Mena and VASP provides compelling evidence that Ena/VASP proteins act as inhibitors of movement under these experimental conditions. The increased motility of MV^{D7} cells over MV^{D7}/EGFP-Mena cells confirms this conclusion and demonstrates that Ena/VASP proteins can act as negative regulators of cell movement in other fibroblastic cell types.

Many of the experiments used here depend upon the

appreciably change volume or extent of spreading during normal movement. The average of the absolute values of positive and negative flow describes a cell's ability to shape change (i.e., the sum of membrane extension and retraction). The centroid positions of the cells were fixed in place during this calculation to derive a value independent of cell translocational speed, an important aspect of cell shape analysis since it separates translocation from protrusion/retraction. When this average flow per unit time was compared across the cell populations tested, a striking positive correlation was observed with translocational speed (Figure 8B). The cells expressing FPPPP-mito moved fastest and changed shape most dramatically over time, while the slow moving EGFP-Mena overexpressing cells and FPPPP-CAAX cells changed shape least. These data indicate that membrane extension and retraction rates are retarded by Ena/VASP proteins, and that this effect correlates with cell speed.

FPPPP motif to recruit specifically all endogenous Ena/VASP proteins through their EVH1 domains. While the same considerations apply to this strategy as they would to any dominant-negative or dominant-inhibitory approach, four observations support our conclusions. First, the EVH1-ligand interaction is highly specific and requires a conserved aromatic residue in the motif (Niebuhr et al., 1997; Carl et al., 1999). Control experiments in which this residue was mutated to an alanine caused no detectable redistribution of Mena or VASP and resulted in cell speeds indistinguishable from parental lines. Second, FPPPP-mito had no effect on the speed of MV^{D7} cells, suggesting that the phenotype induced by this construct depends on Ena/VASP proteins. Third, 4-fold overexpression of Mena caused a phenotype similar to that observed through recruitment of endogenous Mena and VASP to the membrane by FPPPP-CAAX. Finally, MV^{D7} cells, which lack endogenous Ena/VASP proteins, move significantly faster than MV^{D7} cells that express Mena at physiological levels.

Different Populations of Ena/VASP Proteins within Cells

Ena/VASP proteins are present at several locations in fibroblasts: focal adhesions, the leading edge, and diffusely within the cytosol. Presence of distinct pools of Mena and VASP within cells prompts the question: which of these pools are responsible for the observed effects on motility? The three FPPPP-mediated redistribution strategies all deplete Ena/VASP proteins from focal adhesions, but each results in different effects on cell motility that correlate with changes in the leading edge pool. FPPPP-cyto cells, which retain normal membrane-localized Mena and VASP, have motility properties indistinguishable from controls despite absence of Mena and VASP from focal adhesions. This observation, combined with the results of experiments in which membrane recruitment of Mena and VASP by FPPPP-CAAX inhibits motility, is most consistent with a model in which membrane-associated Ena/VASP proteins cause the observed effects on cell speeds. It is interesting to consider the reported strong positive correlation between lamellipodial protrusion rate and fluorescent signal strength of GFP-VASP at the leading edge (Rottner et al., 1999). This observation seems to support a model in which Ena/VASP proteins stimulate protrusion. However, these data are correlative and do not prove direct causation. Perhaps fast moving leading edges harbor more binding sites for Ena/VASP proteins as part of an adaptation or negative feedback mechanism to act as a brake for rapidly expanding lamellipodia.

The results of experiments with FPPPP-cyto cells also indicate that Mena and VASP are not required at focal adhesions for motility under the assay conditions. As judged by staining for five focal adhesion markers, the formation, distribution, and morphology of focal adhesions appeared unaffected by depletion of Mena and VASP. We attempted to perform quantitative cell adhesion assays on the various cell populations using several methods including a centrifugation assay. Unfortunately, these cells were not amenable to such analysis because they adhered so robustly to fibronectin and the other substrata tested that they could not be removed efficiently even by the strongest centrifugation tested. While a quantitative assessment of changes in adhesion was not possible, the lack of a change in speed of FPPPP-cyto cells indicates that focal adhesions retain their ability to support normal motility in the absence of Mena and VASP. Experiments using platelets derived from knockout mice, however, support a role for VASP in regulation of $\alpha_{IIb}\beta_{III}$ integrin binding to fibrinogen (Aszodi et al., 1999; Hauser et al., 1999). It will be important to to test the requirements for Ena/VASP proteins in cells plated on other extracellular matrix proteins or in cell types that express distinct integrin repertoires.

Subcellular Targeting of Ena/VASP Proteins

Recruitment of Ena/VASP proteins to focal adhesions requires direct interactions between the EVH1 domain and proteins containing FPPPP or related motifs such as zyxin and vinculin. However, Rat2 cells expressing sufficient FPPPP-cyto fusion protein to deplete Mena and VASP from focal adhesions have Mena/VASP membrane staining indistinguishable from controls, suggesting that Ena/VASP proteins can be recruited to the leading edge independently of zyxin or other FPPPP-containing proteins in fibroblasts.

Recently, Drees and colleagues used peptide injection to study the role of zyxin in cell motility (Drees et al., 1999). Delocalization of zyxin by microinjection of a peptide that disrupts the interaction between zyxin and α-actinin resulted in a reduction of cell motility. This effect was hypothesized to result from a concomitant delocalization of Ena/VASP proteins along with zyxin from the leading edge. This explanation seems unlikely for two reasons. First, using newly generated monoclonal anti-zyxin antibodies and expression of GFP-zyxin in living cells, we have been unable to detect zyxin at the leading edge under conditions where Mena and zyxin are obviously colocalized in focal adhesions (M. Krause and J. W., unpublished data; J. E. B. and F. B. G., unpublished data). Second, our results indicate a negative role for Ena/VASP proteins in motility and argue for an Ena/VASP-independent explanation for the observed reduction in motility upon zyxin delocalization.

Expression of EGFP-EVH1 demonstrated that the EVH1 domain is sufficient to target GFP signal to the membrane in Rat2 and MV^{D7} cells. This observation, along with the FPPPP-cyto results, suggests that there could be interactions that mediate membrane recruitment of EVH1 domains that do not involve binding to FPPPP motifs. One possibility is that there are other proteins that target Ena/VASP proteins to the leading edge by binding to distinct parts of the EVH1 domain. A second is suggested by the crystal structure of the EVH1 domain (Fedorov et al., 1999; Prehoda et al., 1999). The EVH1 domain resembles the overall structure of pleckstrin homology (PH) domains despite the fact that they share very little sequence identity. PH domains can direct proteins to the membrane through interactions with polyphosphoinositides. It is possible that leading edge targeting of Ena/VASP proteins involves a similar interaction with phospholipids.

Ena/VASP Proteins and the Actin Cytoskeleton

Ena/VASP proteins have been implicated in the direct control of actin polymerization. In vitro, VASP can stimulate actin polymerization and binds directly to F actin via sites in the EVH2 domain (Bachmann et al., 1999; Huttelmaier et al., 1999; Laurent et al., 1999). Ena/VASP proteins also interact directly with profilin, and genetic data in mice support a physiological role for Mena-profilin interactions (Reinhard et al., 1995; Gertler et al., 1996; Lanier et al., 1999). In the context of *Listeria* movement, Ena/VASP proteins have been proposed to accelerate actin filament extension by increasing the local pool of profilin–actin complexes (Beckerle, 1998) although it is clear that Ena/VASP proteins can promote *Listeria* movement independently of profilin (Loisel et al., 1999). Interestingly, experiments using GFP-profilin II demonstrate that profilin is recruited to the surface of motile *Listeria* in an Ena/VASP-dependent manner and accumulation correlates with speed (Geese et al., 2000). While the properties of Ena/VASP:profilin complexes are unknown, binding profilin to a VASP-derived peptide affects the ability of profilin to sequester actin monomer in vitro (Jonckheere et al., 1999). It will be important to determine what role Ena/VASP:profilin interactions play in the context of lamellipodial extension.

Other data implicate Ena/VASP proteins in regulating the actin cytoskeleton in vivo. In contrast to overexpression of VASP, EVL, or Mena, ectopic expression of a larger neuronal-specific isoform of Mena, Mena(+), induces formation of apical, actin-rich extensions in fibroblasts (Gertler et al., 1996). This capacity of Mena(+) to drive formation of abnormal actin-rich structures may result from unique activities contained within the neuronal-specific portion of the protein not found in other family members. It is also possible that Mena(+) is not properly regulated when expressed in nonneuronal cells. Experiments in Jurkat T cells demonstrate that Ena/VASP proteins are also involved in the process of actin cup formation following T cell receptor (TCR) ligation. Expression of soluble GFP-FPPPP repeats disrupts the interaction between Ena/VASP proteins and FYB/SLAP, a component of the TCR signaling complex, blocking actin collar formation following TCR ligation (Krause et al., 2000). Ena/VASP proteins have also been implicated in actin-based cell:cell adhesion. Mena and VASP localize to adherens junctions in epithelial cells. In transgenic mice, expression of a fusion between GFP and part of the VASP EVH2 domain blocks proper formation of adherens junctions in the basal keratinocyte layer of skin, prompting the authors to propose that Mena and VASP promote adhesion zipper formation (Vasioukhin et al., 2000).

How do we reconcile these results with our observations about cell migration and membrane extension? First, actin polymerization cannot simply be equated with cell movement. While cell movement and lamellipodial extension require actin polymerization, they also depend on many other processes. Increased actin dynamics could actually be associated with *reduced* movement, for example, by formation of actin-rich ruffles that do not form productive, extending lamellipodia. Second, the assay we used for Ena/VASP function measured cell movement and membrane extension, not in vivo actin dynamics.

While the exact molecular mechanism of Ena/VASP function remains unclear, it will likely involve regulation of actin dynamics. Ena/VASP proteins might regulate actin polymerization, however, clustering of the endogenous proteins on the mitochondrial surface is not sufficient to cause detectable recruitment or nucleation of F actin. Other accessory molecules or posttranslational modifications of Ena/VASP proteins may be required to regulate actin assembly or binding in vivo. In fact, Ena/VASP proteins might promote or inhibit actin polymerization depending on cellular context.

Ena/VASP Proteins as Negative Regulators of Eukaryotic Cell Motility

Many of the models proposed for the function of Ena/VASP proteins in cell motility draw upon work demonstrating their critical role in promoting the actin-based motility of *Listeria* (Beckerle, 1998; Laurent et al., 1999). Our results indicate that, unlike the actin-based rocketing of *Listeria*, membrane extension and whole cell movement seem to be negatively regulated by Ena/VASP proteins. While *Listeria* is a useful model for many actin-based motility mechanisms, the processes that lead to cell movement likely utilize Ena/VASP proteins in a manner not easily predicted from the study of *Listeria*.

Cell migration is clearly more complicated than *Listeria* movement, and lamellipodial extension involves multiple cellular events that have no part in movement of the bacterium. While a role for Ena/VASP proteins as negative regulators of motility was surprising in light of predictions made based on *Listeria* movement, this inhibitory role is consistent with the results of genetic studies in mice and flies. Platelet aggregation involves dramatic, actin-driven cell shape change. When platelets isolated from VASP-deficient mice are subjected to collagen-induced aggregation assays, they change shape significantly faster than those from wild-type littermates (Aszodi et al., 1999). *Drosophila Ena* mutants show an intersegmental nerve b "bypass" phenotype in which axons move past appropriate choice points and fail to branch (Wills et al., 1999). Axon guidance through such choice points is thought to involve pausing, which requires a decrease in growth cone motility. When paused at choice points, growth cones increase in size and morphological complexity. It is possible that the Ena bypass phenotype reflects a requirement for Ena in growth cone pausing.

Convincing evidence for Ena as negative regulator of growth cone migration comes from the work of Bashaw and colleagues (Bashaw et al., 2000 [this issue of *Cell*]) who report genetic and biochemical interactions between Ena and Robo, a transmembrane axon guidance receptor that associates with Ena through an EVH1-binding site in its cytoplasmic tail. Robo mediates repulsion of axons from the midline in response to its ligand Slit (Kidd et al., 1998, 1999). A series of compelling genetic experiments demonstrate that Ena is a key effector of Robo function. When bound to Slit, Robo may recruit Ena locally to inhibit growth cone advancement towards the midline. This model for Robo-Ena inhibition of growth cone motility is strikingly similar to the effects of membrane recruitment of Ena/VASP proteins on fibroblast motility that we report.

What is the role of Ena/VASP proteins in cell motility? While more work will be required to identify the precise molecular mechanisms by which these molecules regulate cell movement, we postulate that Ena/VASP proteins couple diverse signaling pathways to the control of cell polarity and consequently, migration. Overexpression or constitutive membrane targeting of Ena/VASP proteins inhibits cell movement and appears to block cell polarization. Interestingly, preliminary results suggest that these cells only regain normal polarity and movement immediately following cell division. We speculate that a temporary cell polarity provided by the mitotic machinery may be sufficient to support limited movement in these cells. Such data suggest a role for Ena/VASP proteins in defining or maintaining cell polarity that would put these proteins in a unique position to provide a regulated link between spatially restricted extracellular cues and directional cell movement.

Experimental Procedures

Molecular Cloning
Subcloning and PCR were performed using standard methods. The Mena cDNA (encoding amino acids 1–541) was amplified by PCR and cloned in frame with EGFP (Clonetech) as a C-terminal fusion. This fusion was subcloned into the retroviral vector (pMSCV) to create EGFP-Mena. EGFP-EVH1 is a fusion between EGFP and Mena residues 1–117. Portions of *Listeria* actA were fused with EGFP at the C terminus. An equivalent portion of actA in which the phenylalanine residues in each FPPPP repeat were changed to alanine (Gift of S. Pistor) was used for the APPPP version. FPPPP- and APPPP-mito contain residues 231–610 of ActA. FPPPP- and APPPP-cyto contain residues 231–360 of ActA. The FPPPP- and APPPP-CAAX contain the repeats and a C-terminal fusion to the final 20 residues of human H-ras.

Retroviral Packaging, Infection, FACS Sorting, and Cell Culture
Retroviral packaging and cell culture were performed as described (Gertler et al., 1996). After infection, GFP$^+$ cells were sorted and replated. 24 hr before videomicroscopy, cells were adapted to CO$_2$-independent microscopy media: DME (Gibco/BRL) containing 4500 g/l glucose, 0.35 g/l NaHCO$_3$, 25 mM HEPES, 10% fetal calf serum, and L-glutamine.

Derivation and Culturing of MV^{D7} Cells
E9.5 embryos from Mena/VASP compound heterozygous parents were dissociated by incubation in trypsin/EDTA solution for 25 min in 37°C. To facilitate cell-line derivation, embryos harbored a transgene that expresses a temperature-sensitive version of Large-T antigen. Cells were pelleted, resuspended in Immorto media (DME with 15% FCS, pen/strep, L-glutamine, and 50 U/ml of mouse interferon-gamma [Gibco-BRL]), and plated. After one passage, cells were sorted by FACS into wells of a 96-well plate coated with collagen I (Becton-Dickinson) containing fibroblast-conditioned media. The MV^{D7} line was grown at 32°C in Immorto media.

Immunofluorescence Microscopy
Cells were plated and allowed to spread for 1.5–4 hr on fibronectin-coated coverslips, fixed, and stained as described (Gertler et al., 1996). Anti-Mena (2197) and anti-VASP antisera (2010, raised against murine VASP) were used at 1:400, coumarin-phallicidin (Molecular Probes) was used at 1:20, and anti-vinculin mab (hVin-1, Sigma) was used at 1:400. Secondary antibodies (Texas Red-, Cy5-Donkey anti-rabbit or anti-mouse, Jackson ImmunoResearch) were used at 1:500. Cells were imaged using a Deltavision system (Applied Precision).

Quantitative Western Blots
RIPA lysates were prepared as described (Gertler et al., 1996). Protein content was quantitated using a BCA kit (Pierce). Seven and a half micrograms of lysate was separated by SDS-PAGE and transferred to PVDF membrane. Membranes were incubated with anti-Mena antisera (2197, 1:5000), processed with chemifluorescent reagents (ECF kit, Amersham), and quantitated using a FluorImager and ImageQuant software (Molecular Dynamics). Blots contained a dilution series of purified Mena protein to ensure that signals were within the linear detection range. Fold overexpression numbers represent total Mena signal (endogenous + EGFP-Mena fusion protein) relative to uninfected cells.

Videomicroscopy, Cell Tracking, and Data Analysis
Ten thousand cells adapted to microscopy media were plated on a ΔT dish (Bioptechs) coated with 10 μg/ml fibronectin (Becton Dickinson) and blocked with 1 mg/ml BSA (Sigma); after 1 hr, movies were started. 10× phase movies were 4.5 hr long with frames taken every 5 min using IPLabs software on a Nikon TE300 microscope. Movies were analyzed with DIAS software (Solltech). All cells analyzed remained entirely within the field of view, did not more than transiently touch other cells, and did not divide. Cell paths were generated from centroid positions and parameters (speed and membrane flow) were computed. Speed was calculated using the central difference method in DIAS.

Supplemental Data
Supplemental Videos S1–S3 and Supplemental Table 1 are available online at (http://www.cell.com/cgi/content/full/101/7/717/DC1).

References

Aszodi, A., Pfeifer, A., Ahmad, M., Glauner, M., Zhou, X.H., Ny, L., Andersson, K.E., Kehrel, B., Offermanns, S., and Fassler, R. (1999). The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. EMBO J. *18*, 37–48.

Bachmann, C., Fischer, L., Walter, U., and Reinhard, M. (1999). The EVH2 domain of the vasodilator-stimulated phosphoprotein mediates tetramerization, F-actin binding, and actin bundle formation. J. Biol. Chem. *274*, 23549–23557.

Bashaw, G.J., Kidd, T., Murray, D., Pawson, T. and Goodman, C.S. (2000). Replsive axon guidance: Abelson and Enabled play opposing roles downstream of the Rounabout receptor. Cell, *101*, this issue, 703–715.

Beckerle, M.C. (1998). Spatial control of actin filament assembly: lessons from *Listeria*. Cell *95*, 741–748.

Carl, U.D., Pollmann, M., Orr, E., Gertler, F.B., Chakraborty, T., and Wehland, J. (1999). Aromatic and basic residues within the EVH1 domain of VASP specify its interaction with proline-rich ligands. Curr. Biol. *9*, 715–718.

Chakraborty, T., Ebel, F., Domann, E., Niebuhr, K., Gerstel, B., Pistor, S., Temm-Grove, C.J., Jockusch, B.M., Reinhard, M., Walter, U., et al. (1995). A focal adhesion factor directly linking intracellularly motile Listeria monocytogenes and Listeria ivanovii to the actin-based cytoskeleton of mammalian cells. EMBO J. *14*, 1314–1321.

Domann, E., Wehland, J., Rohde, M., Pistor, S., Hartl, M., Goebel, W., Leimeister-Wachter, M., Wuenscher, M., and Chakraborty, T. (1992). A novel bacterial virulence gene in Listeria monocytogenes

required for host cell microfilament interaction with homology to the proline-rich region of vinculin. EMBO J. *11*, 1981–1990.

Drees, B.E., Andrews, K.M., and Beckerle, M.C. (1999). Molecular dissection of zyxin function reveals its involvement in cell motility. J. Cell Biol. *147*, 1549–1560.

Ermekova, K.S., Zambrano, N., Linn, H., Minopoli, G., Gertler, F., Russo, T., and Sudol, M. (1997). The WW domain of neural protein FE65 interacts with proline-rich motifs in Mena, the mammalian homolog of Drosophila enabled. J. Biol. Chem. *272*, 32869–32877.

Fedorov, A.A., Fedorov, E., Gertler, F., and Almo, S.C. (1999). Structure of EVH1, a novel proline-rich ligand-binding module involved in cytoskeletal dynamics and neural function. Nat. Struct. Biol. *6*, 661–665.

Geese, M., Schluter, K., Rothkegel, M., Jockusch, B.M., Wehland, J., and Sechi, A.S. (2000). Accumulation of profilin II at the surface of Listeria is concomitant with the onset of motility and correlates with bacterial speed. J. Cell Sci. *113*, 1415–1426.

Gertler, F.B., Doctor, J.S., and Hoffmann, F.M. (1990). Genetic suppression of mutations in the Drosophila abl proto-oncogene homolog. Science *248*, 857–860.

Gertler, F.B., Niebuhr, K., Reinhard, M., Wehland, J., and Soriano, P. (1996). Mena, a relative of VASP and Drosophila Enabled, is implicated in the control of microfilament dynamics. Cell *87*, 227–239.

Halbrugge, M., and Walter, U. (1990). Analysis, purification and properties of a 50,000 dalton membrane-associated phosphoprotein from human platelets. J. Chromatogr. *521*, 335–343.

Hauser, W., Knobeloch, K.P., Eigenthaler, M., Gambaryan, S., Krenn, V., Geiger, J., Glazova, M., Rohde, E., Horak, I., Walter, U., and Zimmer, M. (1999). Megakaryocyte hyperplasia and enhanced agonist-induced platelet activation in vasodilator-stimulated phosphoprotein knockout mice. Proc. Natl. Acad. Sci. USA *96*, 8120–8125.

Huttelmaier, S., Harbeck, B., Steffens, O., Messerschmidt, T., Illenberger, S., and Jockusch, B.M. (1999). Characterization of the actin binding properties of the vasodilator-stimulated phosphoprotein VASP. FEBS Lett. *451*, 68–74.

Jonckheere, V., Lambrechts, A., Vandekerckhove, J., and Ampe, C. (1999). Dimerization of profilin II upon binding the (GP5)3 peptide from VASP overcomes the inhibition of actin nucleation by profilin II and thymosin beta4. FEBS Lett. *447*, 257–263.

Kidd, T., Brose, K., Mitchell, K.J., Fetter, R.D., Tessier-Lavigne, M., Goodman, C.S., and Tear, G. (1998). Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. Cell *92*, 205–215.

Kidd, T., Bland, K.S., and Goodman, C.S. (1999). Slit is the midline repellent for the robo receptor in *Drosophila*. Cell *96*, 785–794.

Krause, M., Sechi, A.S., Konradt, M., Monner, D., Gertler, F.B., and Wehland, J. (2000). Fyn-binding protein (Fyb)/SLP-76-associated protein (SLAP), Ena/Vasodilator-stimulated phosphoprotein (VASP) proteins and the Arp2/3 complex link T cell receptor (TCR) signaling to the actin cytoskeleton. J. Cell Biol. *149*, 181–194.

Lanier, L.M., Gates, M.A., Witke, W., Menzies, A.S., Wehman, A.M., Macklis, J.D., Kwiatkowski, D., Soriano, P., and Gertler, F.B. (1999). Mena is required for neurulation and commissure formation. Neuron *22*, 313–325.

Lauffenburger, D.A., and Horwitz, A.F. (1996). Cell migration: a physically integrated molecular process. Cell *84*, 359–369.

Laurent, V., Loisel, T.P., Harbeck, B., Wehman, A., Grobe, L., Jockusch, B.M., Wehland, J., Gertler, F.B., and Carlier, M.F. (1999). Role of proteins of the Ena/VASP family in actin-based motility of Listeria monocytogenes. J. Cell Biol. *144*, 1245–1258.

Loisel, T.P., Boujemaa, R., Pantaloni, D., and Carlier, M.F. (1999). Reconstitution of actin-based motility of Listeria and Shigella using pure proteins. Nature *401*, 613–616.

Niebuhr, K., Ebel, F., Frank, R., Reinhard, M., Domann, E., Carl, U.D., Walter, U., Gertler, F.B., Wehland, J., and Chakraborty, T. (1997). A novel proline-rich motif present in ActA of Listeria monocytogenes and cytoskeletal proteins is the ligand for the EVH1 domain, a protein module present in the Ena/VASP family. EMBO J. *16*, 5433–5444.

Pistor, S., Chakraborty, T., Niebuhr, K., Domann, E., and Wehland, J. (1994). The ActA protein of Listeria monocytogenes acts as a nucleator inducing reorganization of the actin cytoskeleton. EMBO J. *13*, 758–763.

Pistor, S., Chakraborty, T., Walter, U., and Wehland, J. (1995). The bacterial actin nucleator protein ActA of Listeria monocytogenes contains multiple binding sites for host microfilament proteins. Curr. Biol. *5*, 517–525.

Prehoda, K.E., Lee, D.J., and Lim, W.A. (1999). Structure of the enabled/VASP homology 1 domain–peptide complex: a key component of actin assembly. Cell *97*, 471–480.

Reinhard, M., Halbrugge, M., Scheer, U., Wiegand, C., Jockusch, B.M., and Walter, U. (1992). The 46/50 kDa phosphoprotein VASP purified from human platelets is a novel protein associated with actin filaments and focal contacts. EMBO J. *11*, 2063–2070.

Reinhard, M., Giehl, K., Abel, K., Haffner, C., Jarchau, T., Hoppe, V., Jockusch, B.M., and Walter, U. (1995). The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins. EMBO J. *14*, 1583–1589.

Rottner, K., Behrendt, B., Small, J.V., and Wehland, J. (1999). VASP dynamics during lamellipodia protrusion. Nat. Cell Biol. *1*, 321–322.

Schafer, D.A., and Cooper, J.A. (1995). Control of actin assembly at filament ends. Annu. Rev. Cell Dev. Biol. *11*, 497–518.

Schoenwaelder, S.M., and Burridge, K. (1999). Bidirectional signaling between the cytoskeleton and integrins. Curr. Opin. Cell Biol. *11*, 274–286.

Smith, G.A., Theriot, J.A., and Portnoy, D.A. (1996). The tandem repeat domain in the Listeria monocytogenes ActA protein controls the rate of actin-based motility, the percentage of moving bacteria, and the localization of vasodilator-stimulated phosphoprotein and profilin. J. Cell Biol. *135*, 647–660.

Vasioukhin, V., Bauer, C., Yin, M., and Fuchs, E. (2000). Directed actin polymerization is the driving force for epithelial cell–cell adhesion. Cell *100*, 209–219.

Welch, M.D., Rosenblatt, J., Skoble, J., Portnoy, D.A., and Mitchison, T.J. (1998). Interaction of human Arp2/3 complex and the Listeria monocytogenes ActA protein in actin filament nucleation. Science *281*, 105–108.

Wills, Z., Bateman, J., Korey, C.A., Comer, A., and Van Vactor, D. (1999). The tyrosine kinase Abl and its substrate enabled collaborate with the receptor phosphatase Dlar to control motor axon guidance. Neuron *22*, 301–312.