ENa/VASP PROTEINS: Regulators of the Actin Cytoskeleton and Cell Migration

Matthias Krause, Erik W. Dent, James E. Bear, Joseph J. Loureiro, and Frank B. Gertler
Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; email: fgertler@mit.edu

Key Words  Mena, EVL, filopodia, lamellipodia, focal adhesion

Abstract  ENa/VASP proteins are a conserved family of actin regulatory proteins made up of EVH1, EVH2 domains, and a proline-rich central region. They have been implicated in actin-based processes such as fibroblast migration, axon guidance, and T cell polarization and are important for the actin-based motility of the intracellular pathogen Listeria monocytogenes. Mechanistically, these proteins associate with barbed ends of actin filaments and antagonize filament capping by capping protein (CapZ). In addition, they reduce the density of Arp2/3-dependent actin filament branches and bind Profilin at sites of actin polymerization. Vertebrate ENa/VASP proteins are substrates for PKA/PKG serine/threonine kinases. Phosphorylation by these kinases appears to modulate ENa/VASP function within cells, although the mechanism underlying this regulation remains to be determined.

CONTENTS

INTRODUCTION .............................................................. 542
DOMAIN AND FUNCTIONAL ORGANIZATION OF THE ENa/VASP
PROTEIN FAMILY .......................................................... 544
EVH1 Domain .............................................................. 544
Proline-Rich Domain: SH3 and WW Interactions and Profilin Binding .......... 545
EVH2 Domain: Actin Binding and Tetramerization .......................... 545
Isoforms and Unique Features of Mena and EVL .......................... 546
FUNCTIONS OF ENa/VASP PROTEINS IN CELLS AND TISSUES .... 546
Role in Fibroblasts ......................................................... 546
Role in Actin-Based Movement of Bacterial and Viral Pathogens ............. 548
Role in the Immune System: T-Cells and Macrophages ...................... 549
Role in Neuronal Migration and Axon Guidance ................................ 549
Role in Endothelia and Epithelia ........................................ 551
Potential Role During Tumor Formation and Metastasis ....................... 552
MOLECULAR FUNCTIONS OF ENa/VASP PROTEINS ................. 552
REGULATION OF ENa/VASP PROTEINS ......................... 554
Serine/Threonine Phosphorylation ...................................... 554

Copyright © 2003 by Annual Reviews. All rights reserved
First published online as a Review in Advance on July 8, 2003

ENa/VASP PROTEINS: Regulators of the Actin Cytoskeleton and Cell Migration

Matthias Krause, Erik W. Dent, James E. Bear, Joseph J. Loureiro, and Frank B. Gertler
Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; email: fgertler@mit.edu

Key Words  Mena, EVL, filopodia, lamellipodia, focal adhesion

Abstract  ENa/VASP proteins are a conserved family of actin regulatory proteins made up of EVH1, EVH2 domains, and a proline-rich central region. They have been implicated in actin-based processes such as fibroblast migration, axon guidance, and T cell polarization and are important for the actin-based motility of the intracellular pathogen Listeria monocytogenes. Mechanistically, these proteins associate with barbed ends of actin filaments and antagonize filament capping by capping protein (CapZ). In addition, they reduce the density of Arp2/3-dependent actin filament branches and bind Profilin at sites of actin polymerization. Vertebrate ENa/VASP proteins are substrates for PKA/PKG serine/threonine kinases. Phosphorylation by these kinases appears to modulate ENa/VASP function within cells, although the mechanism underlying this regulation remains to be determined.
INTRODUCTION

One of the fundamental problems in biology is to understand how cells move within tissues. Remodeling of the actin cytoskeleton provides the driving force for cell migration. Therefore, it is of great importance to unravel the signaling pathways that regulate the actin polymerization cycle in living cells at the molecular level. Proteins of the Ena/VASP family have emerged as regulators of actin assembly and cell motility in a variety of organisms and cell types for several reasons.

First, Ena/VASP proteins are implicated in the actin-dependent process of axon guidance in Drosophila, Caenorhabditis elegans, and mice. Drosophila Enabled (Ena), one of the founding members of this protein family, was discovered in a genetic screen for dominant suppressors of Drosophila Abelson tyrosine kinase (D-Abl) mutants (Gertler et al. 1990). Hemizygosity for Ena alleviates D-Abl mutant phenotypes. Complete removal of Ena causes defects in the axonal architecture of the central nervous system (CNS) and the peripheral nervous system (PNS) in the fly (Gertler et al. 1995). Much genetic and molecular evidence implicates Ena and unc-34, the C. elegans ortholog of Ena, in the pathways downstream of a number of axon guidance receptors and signaling molecules (Bashaw et al. 2000, Gitai et al. 2003, Wills et al. 1999, Yu et al. 2002). In mice, deletion of mammalian Enabled (Mena), the mammalian Ena ortholog, causes defects in the formation of nerve fiber tracts in the brain (Lanier et al. 1999).

Second, vasodilator-stimulated phosphoprotein (VASP), the other founding member of the Ena/VASP family, was discovered independently as a protein phosphorylated in platelets in response to agents that elevate cAMP and cGMP (Haffner et al. 1995, Halbrugge & Walter 1989, Waldmann et al. 1987). VASP mutant mice exhibit defects in the actin-dependent process of platelet aggregation (Aszodi et al. 1999, Hauser et al. 1999).

Third, Ena/VASP proteins localize within cells to areas of dynamic actin reorganization such as the leading edge of lamellipodia and at the tips of filopodia and other actin-dependent intracellular structures such as cell-cell contacts, focal adhesions, and in periodic puncta along stress fibers (Gertler et al. 1996, Lambrechts et al. 2000, Lanier et al. 1999, Reinhard et al. 1992, Rottner et al. 1999) (Figure 1). In addition, Ena/VASP proteins bind directly to F- and G-actin and the actin monomer-binding protein Profilin, suggesting that they could directly regulate actin dynamics (Ahern-Djamali et al. 1999; Bachmann et al. 1999; Gertler et al. 1996; Huttelmaier et al. 1999; Lambrechts et al. 2000; Laurent et al. 1999; Reinhard et al. 1992, 1995a; Walders-Harbeck et al. 2002).

Fourth, Ena/VASP proteins are recruited to the actin tails formed by Listeria monocytogenes, an intracellular, pathogenic bacterium that uses the host’s actin cytoskeleton to propel itself through the cytoplasm (Chakraborty et al. 1995). This was the first mammalian actin cytoskeletal-associated protein found to be...
TABLE 1 Strategies for modifying Ena/VASP function

<table>
<thead>
<tr>
<th>Method</th>
<th>Organism or cell type</th>
<th>Notes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss-of-function:</td>
<td>Drosophila (ena)</td>
<td>Genetic lesion that causes no protein or nonfunctional protein to be produced</td>
<td>Gertler et al. 1990</td>
</tr>
<tr>
<td>Random mutation</td>
<td>C. elegans (unc-34)</td>
<td></td>
<td>Gitai et al. 2003</td>
</tr>
<tr>
<td>Loss-of-function:</td>
<td>Mouse (mena and vasp), Dictyostelium (Ddvasp)</td>
<td>Homologous recombination gene targeting produces a protein-null mutation</td>
<td>Lanier et al. 1999</td>
</tr>
<tr>
<td>Gene targeting</td>
<td></td>
<td></td>
<td>Aszodi et al. 1999,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hauser et al. 1999,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Han et al. 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Goh et al. 2002</td>
</tr>
<tr>
<td></td>
<td>FPPPPP-Cyto</td>
<td>T cells, fibroblasts, macrophages</td>
<td>Displaces Ena/VASP proteins from interface of T cells with antigen-presenting cells and phagocytic cups of macrophages. In fibroblasts it displaces Ena/VASP proteins from focal adhesions but not from the leading edge. Specificity control: APPPPP-Cyto</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Krause et al. 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Coppolino et al. 2001</td>
</tr>
<tr>
<td></td>
<td>Overexpression:</td>
<td>Fibroblasts</td>
<td>Increased levels of wild-type Ena/VASP protein</td>
</tr>
<tr>
<td></td>
<td>Full-length protein</td>
<td></td>
<td>Bear et al. 2000</td>
</tr>
<tr>
<td></td>
<td>Keratinocytes, mouse skin</td>
<td>Expression of the coiled-coil domain of VASP</td>
<td>Vasioukhin et al. 2000</td>
</tr>
<tr>
<td></td>
<td>Tetramization</td>
<td>Transgenics</td>
<td>Specificity control: none</td>
</tr>
<tr>
<td></td>
<td>Domain (TD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EVH2 domain</td>
<td>Endothelial cells, Fibroblasts</td>
<td>Expression of the EVH2 domain from various family members</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Specificity control: FAB deletion</td>
</tr>
<tr>
<td></td>
<td>Membrane tethering by lipid anchor</td>
<td>Dictyostelium</td>
<td>Direct targeting of DdVASP to the plasma membrane by N-myristolation</td>
</tr>
<tr>
<td></td>
<td>FPPPPP peptide</td>
<td>Neutrophils, Listeria-infected PtK2 cells</td>
<td>Injection or osmotic shock loading of FPPPPP-based peptides Specificity control: irrelevant peptide</td>
</tr>
<tr>
<td>loading/injection</td>
<td></td>
<td></td>
<td>Liu et al. 1999</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>Fibroblasts</td>
<td>Random integration of transcription trap retrovirus produces antisense transcript</td>
</tr>
</tbody>
</table>

Finally, the use of genetic approaches and strategies to interfere with Ena/VASP function (see Table 1) reveals the requirements for Ena/VASP proteins in the developmental and physiological processes of T cell activation, phagocytosis, epithelial morphogenesis, and migration of neutrophils, fibroblasts, and neurons (Anderson et al. 2003; Bear et al. 2000, 2002; Coppolino et al. 2001; Goh et al. 2002; Grevengoed et al. 2001; Krause et al. 2000; Vasioukhin et al. 2000). All the recruited to Listeria, a system used as a model to study the basic principles of the actin cytoskeleton.
above-mentioned processes depend upon regulated cytoskeletal remodeling and implicate Ena/VASP as a key link between signaling pathways and actin dynamics.

DOMAIN AND FUNCTIONAL ORGANIZATION OF THE Ena/VASP PROTEIN FAMILY

The Ena/VASP family consists of Drosophila Ena, C. elegans Unc-34, Dictyostelium DdVASP, and the three mammalian family members VASP, Mena, and EVL (Ena-VASP-like). All Ena/VASP family members share a conserved domain structure. An amino-terminal Ena/VASP homology 1 (EVH1) domain followed by a proline-rich central region and a carboxy-terminal Ena/VASP homology 2 (EVH2) domain.

EVH1 Domain

The EVH1 domain belongs to the PH domain superfamily (Ball et al. 2000, Barzik et al. 2001, Beneken et al. 2000, Fedorov et al. 1999, Prehoda et al. 1999). Unlike PH domains, EVH1 domains do not appear to bind to phosphatidylinositol lipids, although only PI(4,5)P2 has been tested (Volkman et al. 2002). Instead, the EVH1 domain, similar to PTB domains (another branch of the PH domain superfamily), binds to peptide ligands with high affinity. EVH1 domains are also found in the WASP family, the Homer/Vesl family, and two other proteins, Spred and SMIF (Brakeman et al. 1997, Callebaut 2002, Callebaut et al. 1998, Gertler et al. 1996, Kato et al. 1997, Symons et al. 1996, Wakioka et al. 2001). Similar to WW and SH3 domains, the characterized EVH1 domains appear to bind to peptides containing a poly-proline II helix (PPII helix), a left-handed helix with three residues per turn (Ball et al. 2000, Carl et al. 1999, Fedorov et al. 1999, Niebuhr et al. 1997, Prehoda et al. 1999). Flanking residues in the vicinity of the PPII helix confer specificity of binding to SH3, WW, and EVH1 domains. The EVH1 domain in Ena/VASP proteins binds to the consensus site (F/W/Y/L)PPPXX(D/E)(D/E)(D/E)Φ (X = any amino acid, Φ = hydrophobic amino acid). Individual prolines in the central core can be exchanged for F/W/Y and, in some cases, for other amino acids (Ball et al. 2000, Carl et al. 1999, Niebuhr et al. 1997). The other EVH1 domains found in the Homer/Vesl and WASP protein families have binding specificities that differ from those of the Ena/VASP family (Barzik et al. 2001, Beneken et al. 2000, Tu et al. 1998, Volkman et al. 2002, Zettl & Way 2002).

contact site of T cells with antigen-presenting cells by Fyb/SLAP, and to *Listeria* by ActA. Other known proteins such as Palladin, Migfilin, human FAT, human AnkyrinG, and several novel proteins also harbor putative EVH1-binding sites (Mykkänen et al. 2001, Niebuhr et al. 1997, Parast & Otey 2000, Tu et al. 2003; M. Krause & F.B. Gertler unpublished data).

### Proline-Rich Domain: SH3 and WW Interactions and Profilin Binding

The central proline-rich region harbors binding sites for SH3 and WW domain–containing proteins and the actin monomer-binding protein Profilin. This region is the most divergent within the family and therefore may have different binding partners and mechanisms of regulation. Ena binds to the SH3 domains of Abl, Src, and the carboxy-terminal SH3 domain of Drk (Ahern-Djamali et al. 1999, Comer et al. 1998, Gertler et al. 1995). Similarly, EVL binds to the SH3 domains of Lyn, N-Src, Abl, and the WW domain of FE-65 (Lambrechts et al. 2000). In contrast, Mena does not bind to the SH3 domain of N-Src, but is bound by the SH3 domain of IRSp53, Abl, Arg, Src, and the WW domain of FE65 (Ernecova et al. 1997, Gertler et al. 1996, Krugmann et al. 2001). It is not clear which of these interactions is physiologically significant and shared among all members of the Ena/VASP family.

All Ena/VASP family members contain proline-rich-binding sites for the small G-actin-binding protein Profilin, and this binding is independent of their phosphorylation status (Ahern-Djamali et al. 1999, Gertler et al. 1996, Lambrechts et al. 2000, Reinhard et al. 1995b). Profilin II, the major Profilin isoform expressed in brain tissues, binds as a dimer with high affinity to VASP but with low affinity to PI(4,5)P2. In contrast, Profilin I displays the opposite preferences (Jonckheere et al. 1999).

### EVH2 Domain: Actin Binding and Tetramerization

indicate that homo- and heterotetramerization among the members of the Ena/VASP family can also be observed in vivo (Ahern-Djamali et al. 1998, Carl et al. 1999; F.B. Gertler unpublished data). The presence of the coiled-coil within VASP enhances in vitro actin binding and bundling, suggesting that oligomerization facilitates these activities (Bachmann et al. 1999, Walders-Harbeck et al. 2002).

Isoforms and Unique Features of Mena and EVL

Following the EVH1 domain, Mena contains a long insertion not found in other Ena/VASP family members. This insertion in Mena is a striking five-amino acid long stretch of highly charged basic and acidic amino acids (LERER) that are repeated 14 times. This repeat might adopt an extended helical structure that function as a protein-protein-binding interface (Gertler et al. 1996). Mena’s *Drosophila* ortholog Ena contains a glutamine-rich insertion in the same relative part of the molecule with no known function.

At least four different isoforms of Mena can be detected on Western blots of tissue extracts. The smallest isoform of Mena has been found in B-lymphoid cells and arises from the omission of an exon coding for part of the proline-rich region in the Mena 80-kDa isoform (Mena-80). Tani and colleagues described this isoform as 80 kDa and named it Mena-s, but because its apparent molecular mass is clearly smaller than the originally described Mena 80-kDa isoform, we propose to rename it as Mena-75 (Gertler et al. 1996, Tani et al. 2003). The 88-kDa Mena isoform might arise from the inclusion of a known or unknown exon and is expressed in developing tissues (Gertler et al. 1996). All the larger Mena isoforms (Mena-140) contain a large exon that introduces 246 additional amino acids after the LERER repeats. The amino acid sequence introduced by this exon is proline-rich. Some cDNAs containing this exon also include two additional small exons that introduce 4 and 19 amino acids, respectively, directly after the EVH1 domain (Gertler et al. 1996). Mena-140 is expressed in developing and adult neuronal tissues (Lanier et al. 1999) and is tyrosine phosphorylated in vivo (Gertler et al. 1996). EVL is expressed in two isoforms that migrate differently in SDS-gels (Lambrechts et al. 2000). The larger isoform, EVL-I, arises from an alternative exon coding for 21 amino acids spliced into the EVH2 domain just before the coiled-coil motif.

FUNCTIONS OF Ena/VASP PROTEINS IN CELLS

AND TISSUES

Role in Fibroblasts

Fibroblast migration is a complex process in which forward protrusion, attachment, contraction, and rear detachment are integrated to translocate the cell. To study the role of Ena/VASP proteins in fibroblast motility, cell lines were derived from mouse embryos devoid of Mena and VASP. One fibroblastic cell line from this collection that lacked expression of all Ena/VASP proteins (including the third
mammalian family member, EVL) was analyzed by video microscopy. When this cell line (the MV\textsuperscript{D7} cells) is rescued with a physiological level of Mena, EVL, or VASP, the cells moved more slowly than the parental null line. Conversely, the overexpression of Ena/VASP proteins in normal fibroblasts led to slower movement (Bear et al. 2000).

Rescue of the fibroblasts devoid of Ena/VASP proteins with deletion mutants of Mena allowed the structural requirements of Ena/VASP proteins in fibroblast motility to be examined (Loureiro et al. 2002). Re-expression of a mutant lacking the proline-rich region slowed cells down to the same extent as the wild-type protein, indicating that Profilin and other ligand recruitment mediated by this region are dispensable for random cell migration. However, the proline-rich region is required for other Ena/VASP-dependent processes (see below). The F-actin-binding site is essential for proper fibroblast motility and for the ability of Ena/VASP proteins to be recruited effectively to the leading edge.

An alternate approach to block Ena/VASP function is to remove the proteins from their normal localization within cells. As noted above, the EVH1 domain of Ena/VASP proteins binds strongly to the peptide motif \((D/E)FPPPXD/E(D/E)\). This interaction can be exploited to sequester Ena/VASP proteins on the surface of mitochondria by expression of a targeting construct (called FPPPP-Mito, see Table 1) that contains four copies of the FPPPP motif linked to a mitochondrial anchoring sequence. FPPPP-Mito blocks the function of all Ena/VASP family members by deleting them from their normal sites of function and replicates the phenotypes observed in Ena/VASP-deficient cells (Bear et al. 2000). Expression of the FPPPP-Mito construct in the null fibroblasts caused no change in cell speed, confirming the specificity of this approach. Taken together, these observations using the null cells and re-localization strategies led to the conclusion that Ena/VASP proteins negatively regulate fibroblast migration speed.

When Ena/VASP proteins are selectively de-localized from focal adhesions, but not from the leading edge (using FPPPP-Cyto, see Table 1), random fibroblast migration speed on a fibronectin substrate is not affected, indicating that Ena/VASP proteins do not have a critical function in focal adhesions in this type of migration assay. At a gross level, the composition, number, and morphology of focal adhesions is unaffected by loss of Ena/VASP (Bear et al. 2000). At present, the function of Ena/VASP proteins within focal adhesions is unclear. It will be important to determine whether Ena/VASP proteins are required for focal adhesion function in other cell types or when cells are plated on substrates other than fibronectin.

The function of Ena/VASP proteins as negative regulators of fibroblast motility (Bear et al. 2000) seemed paradoxical given that lamellipodial protrusion rate positively correlates with the intensity of GFP-VASP at the leading edge (Rottner et al. 1999). A careful examination of the dynamics of lamellipodia lacking Ena/VASP proteins resolved this apparent conflict and indicated that such lamellipodia protrude more slowly, but the protrusion persists longer. Conversely, cells overexpressing Ena/VASP proteins have lamellipodia that protrude faster than controls, but these protrusions are rapidly withdrawn in the form of ruffles. Therefore,
Ena/VASP proteins increase lamellipodial protrusion velocity but have a global negative effect on cell motility. These observations have led to a more general conclusion that whole-cell motility rates correlate better with persistence of protrusion than they do with instantaneous protrusion rate (Bear et al. 2002).

Interestingly, deletion or re-localization of Ena/VASP proteins from the leading edge causes reorganization of actin filament architecture in lamellipodia (Bear et al. 2002). Lamellipodia lacking Ena/VASP function contain actin filament networks with shorter and more highly branched actin filaments. Excess Ena/VASP leads to longer but less-branched filaments. These changes in actin network geometry are responsible for the changes in lamellipodial behavior and, ultimately, changes in cell migration rate. Modifying Ena/VASP function in fibroblasts has led to a molecular model of Ena/VASP function that is discussed below.

Role in Actin-Based Movement of Bacterial and Viral Pathogens

Like several other bacteria and viruses, Listeria monocytogenes is able to invade mammalian cells and use the host cell’s actin cytoskeleton to move within and from cell to cell. Listeria requires only one bacterial surface protein, ActA, for its intracellular motility (Domann et al. 1992, Kocks et al. 1992). ActA functions as a nucleator of F-actin (Pistor et al. 1994) by co-opting the host cells own Arp2/3 complex for F-actin nucleation (Pistor et al. 2000; Skoble et al. 2000; Welch et al. 1997, 1998). The finding that VASP was recruited to and directly binds, through its EVH1 domain, to proline-rich repeats within ActA was the first indication that Ena/VASP proteins might directly regulate actin dynamics (Chakraborty et al. 1995, Gertler et al. 1996, Niebuhr et al. 1997, Pistor et al. 1995). Deletion of these proline-rich repeats within ActA led to a reduction of intracellular speed and reduced pathogenicity (Lasa et al. 1997, Niebuhr et al. 1997, Smith et al. 1996). As mentioned above, Ena/VASP proteins bind directly in vitro to Profilin, suggesting that the recruitment of Profilin by Ena/VASP proteins may be one function of this protein family important for Listeria motility. This view is supported by the reduction of Listeria speeds after depletion experiments of either Ena/VASP proteins or Profilin from cell extracts and by the restoration of Listeria movement in both add-back experiments and from reconstitution of pure proteins (Theriot et al. 1994, Kang et al. 1997, Laurent et al. 1999, Loisel et al. 1999). Ena/VASP proteins also facilitate ActA-induced Arp2/3-mediated actin nucleation, possibly by increasing the amount of actin monomer available on the bacterial surface (Skoble et al. 2000). Finally, Ena/VASP proteins decrease the number of F-actin branches induced by ActA and the Arp2/3 complex in vitro, suggesting that they might regulate the geometry of the actin filament network in the tails of motile bacteria (Skoble et al. 2000).

Within living cells, Listeria speeds are reduced an order of magnitude in the absence of Ena/VASP proteins. Expression of Ena/VASP deletion mutants lacking either Profilin or the G-actin-binding site in Ena/VASP null cells provided only a
modest increase in bacterial speed relative to wild-type protein, indicating that both
domains are important for *Listeria* motility. In contrast, deletion of the F-actin-
binding site increased the *Listeria* speed to higher than wild-type levels, whereas
deletion of the coiled-coil domain had no effect (Geese et al. 2002). Because the
same set of mutants yielded different results when assayed for function in whole-
cell motility (described above), the molecular requirements for Ena/VASP function
in ActA-mediated bacterial movement likely differ in some important respects from
their function in regulating lamellipodial dynamics. Therefore, it is important to
consider that Ena/VASP proteins probably fulfill different functions depending on
the cellular context, indicating that results obtained assaying a particular function
may not be entirely translatable to other systems (Loureiro et al. 2002). Ena/VASP
proteins are also localized to the F-actin tail or to surfaces of other intracellular
pathogens such as *Shigella flexneri*, *Rickettsia*, and *Vaccinia* virus, but the role, if
any, of Ena/VASP in the motility of these pathogens is unclear (Chakraborty et al.

**Role in the Immune System: T-Cells and Macrophages**

The physiological function of the immune system depends on dynamic changes
of the actin cytoskeleton (Penninger & Crabtree 1999). Actin polymerization is
required for the interaction of T cells with antigen-presenting cells, phagocytosis
by macrophages, and chemotaxis of neutrophils toward bacteria.

Ena/VASP proteins co-localize with the hematopoietic-specific adaptor protein
Fyb/SLAP; the adaptor molecules SLP-76, Nck, Vav, WASP; and the F-actin nu-
cleator Arp2/3 complex in phagocytic cups of macrophages. They also co-localize
at the interface between T cells and anti-CD3 coated beads (to mimic antigen-
presenting cells) (Coppolino et al. 2001, Krause et al. 2000). This localization
of Ena/VASP is mediated by direct binding of the EVH1 domain to Fyb/SLAP
(Krause et al. 2000). Ena/VASP proteins are also detected in a protein complex with
Fyb/SLAP, SLP-76, Nck, Vav, and the Arp2/3 activator WASP upon ligation of the
T cell receptor or the Fc{\gamma} receptor of macrophages, indicating that two key mod-
ulators of the actin dynamics, Ena/VASP and WASP, can be linked in regulated,
physiological processes (Coppolino et al. 2001, Krause et al. 2000). Importantly,
properly localized Ena/VASP proteins and Arp2/3 are each required for effective
Jurkat T cell polarization and for phagocytosis (Coppolino et al. 2001, Krause et al.
2000, May et al. 2000), suggesting that Ena/VASP-WASP-Arp2/3 networks may
function together to promote these processes. Recently, peptide-loading exper-
iments of neutrophils with putative Ena/VASP function-blocking peptides were
described (Anderson et al. 2003) that resulted in reduced migration velocity of
neutrophils (see Table 1).

**Role in Neuronal Migration and Axon Guidance**

The development of the nervous system involves extensive migration of neurons.
To study the role of Ena/VASP proteins in neuronal migration, the FPPPP-Mito
approach described above was used to block Ena/VASP function in cortical neurons (Goh et al. 2002). Neutralization of Ena/VASP function within neurons that normally migrate radially from their birthplace to the deep layers of the cortex resulted in frequent misplacement of these neurons to more superficial layers. Analysis of early stages of neuronal migration indicated that neurons expressing the FPPPP-Mito construct were already present in more superficial structures and therefore moved farther than control cells. These data suggest that Ena/VASP proteins play a key role in neuronal migration, although their effects on cell speed and mode of migration in cortex remain to be determined.

During development, growth cones, the motile tips of growing processes, must traverse great distances and continually integrate a plethora of extracellular guidance signals into appropriate changes in cytoskeletal dynamics for proper movement. There are several reasons to believe that Ena/VASP proteins play important roles in intracellular signaling cascades that transduce extracellular guidance signals into actin dynamics within growth cone filopodia and lamellipodia. First, Ena/VASP proteins are concentrated at the tips of growth cone filopodia and lamellipodia in primary cultured neurons (Figure 2) (Lanier et al. 1999). Second, all three vertebrate family members are regulated by protein kinase A (PKA), and at least VASP is known to be phosphorylated by protein kinase G (PKG) (Aszodi et al. 1999, Gertler et al. 1996, Halbrugge et al. 1990, Lambrechts et al. 2000, Loureiro et al. 2002). PKA and PKG are two important kinases that modulate signaling downstream of many axon guidance molecules (Song & Poo 1999). Third, genetic studies in worms and flies implicate their Ena/VASP homologs in response to specific axonal guidance signals. In worms, *unc-34* is required for proper response to netrin, a guidance factor that can act either as an attractant or a repellent depending on the receptor repertoire and signaling context present within responding neurons (Colavita & Culotti 1998, Gitai et al. 2003). In both flies and worms, Ena/VASP appears to function downstream of the repulsive guidance receptor Robo/Sax3, a molecule that binds directly to Ena/VASP through an EVH1-binding site found on its cytoplasmic tail (Bashaw et al. 2000, Yu et al. 2002). Genetic data also suggest that *Drosophila* Ena and D-ABL may act antagonistically downstream of certain axon guidance receptors (Bashaw et al. 2000, Wills et al. 1999). Other evidence suggests D-lar, a receptor tyrosine phosphatase that antagonizes D-ABL function in peripheral nervous system guidance (Wills et al. 1999), may be linked to Ena function. Deletion of either Ena or D-lar induces similar phenotypes in which intersegmental nerve b (ISNb) peripheral nerves fail to branch at the correct position and instead bypass their muscle target and extend beyond their normal branching point (Wills et al. 1999). Potential regulation of Ena by D-ABL and D-lar are discussed below.

In mice, deletion of Mena caused axonal guidance defects in the formation of the corpus callosum, hippocampal commissure, and pontocerebellar fiber bundles (Lanier et al. 1999). Because all vertebrate Ena/VASP family members are expressed in the developing vertebrate central nervous system (Lanier et al. 1999), it is likely that the continued presence of EVL and VASP in the nervous system
of Mena mutants permits normal formation of other nerve connections that could utilize EVL and/or VASP for their guidance. Studies of compound mutant animals or the use of inhibitory approaches will be required to uncover the full role of Ena/VASP function in nervous system development and specifically in axon guidance. It will be particularly interesting to determine whether vertebrate Ena/VASP proteins play a role in conveying the PKA and PKG-dependent responses from guidance receptors to the cytoskeleton.

Role in Endothelia and Epithelia

Endothelial cells line blood vessels and provide both a barrier function between blood and other tissue while still allowing immune cells access to the surrounding tissue. This barrier function is maintained through tightly controlled cell-cell adhesions and is positively regulated by PKA. Interestingly, phospho-VASP has been localized to cell-cell junctions and could be co-immunoprecipitated with the tight-junction marker zonula-occludens-1 (ZO-1) protein from endothelial cells (Comerford et al. 2002).

Similarly, by adhering to each other, epithelial cells form a physical boundary capable of regulating the transfer of molecules across the epithelial sheet. This permits selective exchange of molecules between the organism and its environment, exemplified in the waste removal function of the kidney or nutrient uptake in the gut. In these systems Ena/VASP proteins localize to adherens junctions, and disruption of their function affects epithelial dynamics (Grevengoed et al. 2001, Lawrence et al. 2002, Reinhard et al. 1992, Vasioukhin & Fuchs 2001). Furthermore, VASP can be co-immunoprecipitated with the tight junction protein ZO-1 from epithelial cells, and blocking PKA phosphorylation reduces the barrier function of epithelial sheets as measured by electrical conductivity across the epithelial barrier (Lawrence et al. 2002).

Studies in Drosophila have demonstrated that reduction of either D-Abl or Ena affects epithelial morphology and epithelial sheet migration. Genetic interactions suggest that Ena functions in concert with components of the cadherin-catenin complex (which mediates cell-cell adhesion) and D-Abl in epithelial sheets at the adherens junction (Grevengoed et al. 2001). In mammals, Ena/VASP proteins are found in epithelial contacts that are revealed as a double row of dot-like structures also containing F-actin, Cadherin, Zyxin, and Vinculin. These cell-cell contacts have been suggested to represent an early stage in cell-cell contact formation and have been termed the adhesion zipper (Vasioukhin et al. 2000). It has been proposed that cell-cell contacts are generated after first contact of lamellipodia and/or filopodia of opposing cells (Ehrlich et al. 2002, Vaezi et al. 2002). Overexpression of a fragment of Ena/VASP responsible for oligomerization (see Table 1) disrupts epithelial sheet formation (Vasioukhin et al. 2000). However, the specificity of this approach for Ena/VASP proteins has not been demonstrated. Although it seems likely that Ena/VASP proteins play an important role in epithelial dynamics, further work will be required to clarify their role in such processes.
Potential Role During Tumor Formation and Metastasis

Up- or down-regulation of Ena/VASP proteins has not been decisively linked to tumor progression in humans. However, it has been reported that either increasing (by overexpression) or decreasing (by antisense transcriptional knockdown, see Table 1) levels of VASP protein in NIH 3T3 fibroblasts may cause a tumorigenic phenotype when these cells have been injected into nude mice (Liu et al. 1999). This observation is curious given that Mena, VASP, and EVL have broad overlapping expression patterns and exhibit extensive functional overlap in vitro and in vivo. Furthermore, a similar phenotype has not been reported for VASP or Mena knockout mice, which are adult-viable when individually mutated. Given that Ena/VASP proteins affect the mitogenic properties of cells, it is tempting to speculate that loss-of-Ena/VASP-function potentiates metastasis. However, further studies are necessary to clarify the role of Ena/VASP, if any, during tumorigenesis.

MOLECULAR FUNCTIONS OF Ena/VASP PROTEINS

How can the Ena/VASP-dependent phenotypes seen in fibroblast migration, *Listeria* motility, and other actin-dependent processes be explained at the molecular level? Early models of Ena/VASP function suggested a role in the de novo nucleation of actin filaments. Like many other actin-binding proteins, purified Ena/VASP proteins can shorten the lag phase of actin polymerization in in vitro assays (Bachmann et al. 1999, Huttelmaier et al. 1999, Lambrechts et al. 2000, Laurent et al. 1999). However, no convincing evidence supports this as a physiological mechanism for Ena/VASP function in vivo. One report showed that Ena/VASP proteins targeted to the mitochondria could recruit F-actin, supposedly through nucleation of new filaments (Fradelizi et al. 2001). However, these experiments used permeabilized cells and added exogenous actin. Several other laboratories failed to observe F-actin recruitment when Ena/VASP proteins were relocalized to mitochondria in intact cells. Furthermore, *Listeria* that exhibit defective Arp2/3 activation but still recruit normal levels of Ena/VASP proteins fail to assemble or recruit any detectable F-actin (Bear et al. 2000; Lasa et al. 1997; Pistor et al. 1994, 1995; Skoble et al. 2000). More recent experiments both in vivo and in vitro point to three likely molecular mechanisms of Ena/VASP function: anti-capping, anti-branching, and Profilin recruitment.

Ena/VASP proteins may act to prevent or delay capping of barbed ends of actin filaments by CapZ. Multiple lines of evidence support this anti-capping mechanism. Ena/VASP proteins are localized to the leading edge of lamellipodia and the tips of filopodia. These locations contain a high density of actin filament barbed ends, and Ena/VASP localization to these places can be displaced by treatment with low concentrations of cytochalasin D (CD) (Bear et al. 2002). This drug binds with high affinity to the barbed ends of actin filaments and presumably displaces Ena/VASP proteins by competition. This observation is supported by in vitro assays in which Ena/VASP-coated beads can capture uncapped, but not capped,
Ena/VASP function may also act to inhibit the process of Arp2/3-mediated actin filament branching or to reduce branch stability. Results from in vitro experiments showed that VASP decreases the number of F-actin branches induced by ActA from *Listeria* and the Arp2/3 complex (Skoble et al. 2001). Another study, however, saw no effect of VASP on branching (Boujemaa-Paterski et al. 2001). In support of the work from Skoble and co-workers, modification of Ena/VASP activity in fibroblasts led not only to changes in filament length but also to changes in branching density. High Ena/VASP activity was associated with low branching density, whereas low Ena/VASP activity led to high branching density. The association of Ena/VASP proteins with actin filaments may either compete with Arp2/3 for binding sites near the barbed end or condition the filaments in a way to inhibit branching or to promote debranching. Future experiments will be required to understand the anti-branching activity of Ena/VASP proteins on the molecular level. Interestingly, the rescue of the fibroblast motility phenotypes by low doses of CD selectively affected filament length, but not branching. This suggests that changes in length alone may explain the fibroblast motility results (Bear et al. 2002).

Ena/VASP function may also involve binding to Profilin at sites of actin reorganization. Genetic experiments in mice indicate that Mena mutants are sensitive to a 50% reduction in Profilin I levels (Lanier et al. 1999). Normally, Profilin I heterozygotes are viable despite a twofold reduction in Profilin levels. When bred into a Mena mutant background, heterozygosity for Profilin I caused Mena mutants to die perinatally and suffer neurulation defects. This genetic experiment indicates that Mena and Profilin I are both involved in the actin-dependent process of neural tube closure. Ena/VASP proteins bind directly to Profilin in vitro. In vitro assays also show that a peptide containing the Profilin-binding site from VASP affects the biochemical properties of Profilin II, permitting actin nucleation to occur in the presence of the actin-sequestering protein, thymosin beta-4, which would otherwise block actin nucleation when combined with Profilin (Jonckheere et al. 1999).

As noted above, Profilin recruitment by Ena/VASP plays an important role in supporting *Listeria* motility within living cells. EGFP-Profilin co-localizes only with moving *Listeria*, and its intensity correlates positively with the speed of the actin filaments. In addition, purified VASP antagonizes the activity of CapZ in actin filament elongation assays. Perhaps the most compelling evidence for this model comes from studies of fibroblasts with modified Ena/VASP function. Depletion of Ena/VASP leads to shorter actin filaments at the leading edge relative to controls. Conversely, overexpression of Ena/VASP leads to longer than normal filaments at the leading edge (Bear et al. 2002). These data suggest a mechanism in which Ena/VASP proteins associate with actin filaments at or near their barbed ends to prevent capping by CapZ. This model is supported by experiments in vivo in which low doses of CD were used to reverse Ena/VASP-dependent motility phenotypes in fibroblasts. This CD treatment mimics CapZ overexpression and reduced the filament length to wild-type levels, indicating that a balance between CapZ levels and Ena/VASP activity regulates actin filament geometry in lamellipodia (Bear et al. 2002).
bacteria (Geese et al. 2000). In addition, injection of cross-linked Profilin-actin (which inhibits the elongation rate of actin filaments in vitro) into \textit{Listeria}-infected cells reduces bacterial speed. A mutant version of this cross-linked Profilin-actin complex that cannot bind to Ena/VASP proteins does not reduce speed (Grenklo et al. 2003). Consistent with this model, Ena/VASP-deficient cells that are complemented with forms of Mena or VASP that cannot bind Profilin (the proline-rich deletion) do not support \textit{Listeria} movement as effectively as wild-type protein (Geese et al. 2002). However, this same experiment indicates that Ena/VASP proteins play additional roles in \textit{Listeria} motility that are independent of Profilin recruitment. Furthermore, Profilin-binding-defective mutants of Mena fully support normal fibroblast movement, indicating that this process does not require Profilin recruitment by Ena/VASP. Cellular processes other than random fibroblast migration may also be dependent on an Ena/VASP-dependent Profilin recruitment (e.g., filopodia extension or phagocytosis).

What molecular role might Ena/VASP proteins play in other cellular processes such as filopodial formation? Recently, Svitkina and colleagues showed that filopodia arise from the bundling of preexisting filaments within the actin filament network of lamellipodia, rather than from de novo actin filament nucleation. Interestingly, GFP-VASP was identified as an early marker of filopodial precursors. Recruitment of VASP was followed by subsequent recruitment of the F-actin-bundling protein, fascin, and filopodia formation (Svitkina et al. 2003). In another study, a knockout of the sole Ena/VASP family protein in \textit{Dictyostelium}, DdVASP, resulted in the absence of filopodia, providing evidence that Ena/VASP proteins are required for filopodia formation (Han et al. 2002). In vitro reconstitution of filopodia with purified proteins or cellular extracts demonstrates that lowering the amount of CapZ can transform a dendritic network into a bundled filopodia-like structure (Vignjevic et al. 2003). According to this model, the anti-capping activity of Ena/VASP would be expected to facilitate filopodial formation.

**REGULATION OF Ena/VASP PROTEINS**

**Serine/Threonine Phosphorylation**

The mammalian Ena/VASP proteins are known substrates of cAMP- and cGMP-dependent serine and threonine kinases, PKA and PKG, respectively (Figure 3) (Gertler et al. 1996, Lambrechts et al. 2000, Waldmann et al. 1987). VASP harbors three phosphorylation sites (Ser-157, Ser-239, and Thr-278), whereas Mena contains the first two, and EVL only the first site (Butt et al. 1994, Gertler et al. 1996, Lambrechts et al. 2000). Phosphorylation of Ser-157 of VASP leads to a shift in apparent molecular mass in SDS-PAGE from 46 to 50 kDa, indicating that this phosphorylation may cause a change in secondary structure of the molecule (Halbrugge & Walter 1989). Phosphorylation of Mena and EVL at the position that is equivalent to VASP Ser-157 also induces bandshifts in their mobility (Gertler et al. 1996, Lambrechts et al. 2000).
Ena/VASP FUNCTION

For VASP, PKG initially phosphorylates the second site then the first, and finally the third site in vitro. Conversely, PKA phosphorylates, in vitro and in intact platelets, the first, second, and third sites in order (Butt et al. 1994, Halbrugge et al. 1990). However, in vivo PKG appears to phosphorylate VASP with different kinetics: The first two sites are phosphorylated in intact platelets with a similar time course but only 50% of VASP shifts from 46 to 50 kDa (Butt et al. 1994).

PKA/PKG phosphorylation of VASP at the various conserved sites has been observed in response to a variety of physiological stimuli (Walter et al. 1993). In platelets, for example, inhibition of fibrinogen binding to the fibrinogen receptor $\alpha_{IIb}$$\beta_{3}$ integrin correlates with VASP phosphorylation in platelets, suggesting a role for VASP in the regulation of fibrinogen receptor activation (Halbrugge et al. 1990, Horstrup et al. 1994, Waldmann et al. 1987). Studies of mutant mice revealed that VASP is required for the PKA-mediated inhibition of platelet aggregation (Aszodi et al. 1999, Hauser et al. 1999), suggesting that VASP is the critical PKA substrate required for this process, and that Ser-157 may be the key site of regulation.

VASP is also phosphorylated by PKA on its first phosphorylation site upon detachment of fibroblasts and is dephosphorylated transiently during reattachment to fibronectin. During subsequent cell spreading, VASP becomes heavily phosphorylated again (Howe et al. 2002). Whether such phosphorylation reflects the overall phosphorylation of all VASP within the cell or whether VASP at the leading edge and VASP at focal adhesions are differently phosphorylated remains an important yet unsolved question.

In random fibroblast migration, phosphorylation of Mena at its first phosphorylation site (S-236, the equivalent of VASP Ser-157) is essential for its function. A non-phosphorylatable S-236-A Mena mutant localizes normally when expressed in Ena/VASP-deficient cells, yet fails to rescue the hypermotile phenotype of Ena/VASP null cells (Loureiro et al. 2002). Conversely, a phosphomimetic S-236-D Mena mutant complements the fibroblast phenotype. Therefore, it seems likely that phosphorylation is not essential for Ena/VASP subcellular targeting but is probably required to regulate Ena/VASP function in lamellipodia. This raises the interesting possibility that Ena/VASP activity might be regulated by the function of AKAPs, proteins that anchor and regulate PKA function in discrete subcellular locations (Diviani & Scott 2001). Taken together, these experiments suggest that PKA phosphorylation of vertebrate Ena/VASP proteins, particularly at the first site, likely elevates their activity.

Interestingly, *Drosophila* Ena lacks a clear equivalent of VASP Ser-157 (Figure 3) (Gertler et al. 1996). When expressed in Ena/VASP-deficient cells, Ena localizes properly but fails to complement the hypermotility phenotype, possibly because it cannot be phosphorylated by PKA (Loureiro et al. 2002). The isolated EVH2 domains of both Mena and Ena can localize to lamellipodia, though in a broader pattern than the intact protein. The EVH2 domain alone can complement the hypermotile phenotype of Ena/VASP-deficient cells. Together, these results indicate that the EVH2 domain contains the functional elements required to regulate
lamellipodial dynamics and suggest that the rest of the protein (including Ser-236 of Mena) likely plays a regulatory role. The fact that the Ena EVH2 functions in this assay suggests that it can perform the molecular functions required to regulate lamellipidial dynamics, but that the intact molecule is inactive, perhaps because the Ser-157/-236 equivalent is absent. If correct, this would suggest that PKA regulation of Ena/VASP proteins evolved in the vertebrate system after the divergence with invertebrates. Interestingly, VASP can largely rescue the *Drosophila Ena* mutant phenotype, again suggesting that the basic molecular functions of the family have been conserved across evolution but that the vertebrate proteins may require additional regulation by PKA/PKG.

What is the effect of serine/threonine phosphorylation on Ena/VASP function? Because phosphorylation of both Ser-157 in VASP and Ser-236 in Mena appears to correlate with their activity in various processes, it seems likely that this phosphorylation relieves either an intra- or intermolecular inhibitory interaction. More work is required to determine the mechanism by which phosphorylation activates Ena/VASP function.

It is known that binding of proteins to the EVH1 domain occurs independently of phosphorylation (Harbeck et al. 2000). In contrast, binding of certain SH3-domain-containing proteins to the proline-rich central region of Ena/VASP proteins is regulated by serine/threonine phosphorylation. Serine/threonine phosphorylation of EVL by PKA disrupts the binding of EVL to the SH3 domain of c-Abl and nSrc. However, PKA phosphorylation had no effect on the binding of the SH3 domain of Lyn and the WW domain of FE-65 to EVL, and of Profilin to VASP (Harbeck et al. 2000, Lambrechts et al. 2000). This is consistent with a recent report that VASP forms a complex with c-Abl only when VASP is not phosphorylated (Howe et al. 2002).

In the EVH2 domain, the binding to G-actin is greatly reduced by phosphorylation of the second serine site in VASP (Walders-Harbeck et al. 2002). This phosphorylation site is conserved in Mena but absent in EVL, which points to differences among the family members in regard to their regulation (Gertler et al. 1996). The effect of phosphorylation by PKA on F-actin-binding and -bundling activity is unclear at present because two studies that used different salt concentrations in their buffers came to opposite conclusions (Harbeck et al. 2000, Laurent et al. 1999).

**Tyrosine Phosphorylation**

* *Drosophila Ena* was originally identified as a suppressor of lethality and CNS defects associated with *D-Abl* mutant phenotypes (Gertler et al. 1990). Furthermore, Ena is phosphorylated by D-Abl on multiple tyrosine residues in vitro and when co-expressed in cells (Comer et al. 1998, Gertler et al. 1995). Ena phosphorylation is decreased in *Abl* mutant flies, indicating that Abl phosphorylates Ena during development. However, this also suggests that Ena may be phosphorylated by other tyrosine kinase(s) (Gertler et al. 1995).
What is regulated by tyrosine phosphorylation of Ena? The phosphorylated tyrosine residues are clustered in the proline-rich region (Figure 3). Consistently, it has been shown that the binding of the SH3 domain of Abl and Src to Ena is abrogated when it is phosphorylated by D-Abl in vitro (Comer et al. 1998). However, at present it is unclear whether tyrosine phosphorylation of Ena is important for its function. Expression of a non-phosphorylatable mutant of the six major sites of Ena in Ena mutant flies partially rescued viability (Comer et al. 1998). The same mutant was still capable of rescuing the bypass phenotype in the ISNb peripheral nerve seen in the Drosophila Ena mutant (D. Van Vactor, personal communication). Furthermore, human VASP, which is not phosphorylated on tyrosine residues, was able to rescue the embryonic lethality associated with loss of Ena function in Drosophila (Ahern-Djamali et al. 1998). The basis for Ena suppression of D-Abl phenotypes remains unclear. It is interesting to note that Abl-family proteins can bind F- and G-actin and that D-Abl also contains EVH1-binding sites, suggesting that mechanisms other than D-Abl phosphorylation of Ena could be responsible for the observed genetic interactions.

One difference between vertebrate and invertebrate Ena/VASP proteins is that the tyrosine phosphorylation sites in Ena are not conserved in the mammalian Ena/VASP proteins (Figure 3). The neuronal-specific Mena-140 isoform has been shown to be tyrosine phosphorylated in vivo (Gertler et al. 1996). Recently, it was demonstrated that Mena-75 and Mena-80 can also be phosphorylated by c-Abl at Tyr-296 when co-expressed with Abi-1 and c-Abl in cells (Tani et al. 2003). Whether this phosphorylation occurs with endogenous levels of c-Abl, Abi-1, and Mena is unknown. Mena Tyr-296 is not conserved in VASP or EVL, raising the possibility that only Mena, but not EVL or VASP, is regulated by c-Abl. Tyr-296 in Mena is localized close to the proline-rich central region, as it is in Ena, suggesting that this phosphorylation could regulate binding of ligands to Mena.

**FUTURE DIRECTIONS**

Although a great deal has been learned about Ena/VASP proteins since they were discovered, a number of unanswered questions remain. One important question is whether the mammalian Ena/VASP proteins are truly functionally interchangeable or whether they have evolved some unique roles. In fibroblast motility and Listeria movement, they function equivalently. However, some cell types or subcellular processes may utilize the family members in unique ways. For example, it was reported recently that cardiac fibroblasts derived from VASP knockout mice, which express normal levels of Mena and EVL, showed increased spreading, prolonged Rac and PAK activity, and cell migration defects (Garcia Arguinzonis et al. 2002). The analysis of compound knockouts in all three murine genes should unravel unique functions for the different Ena/VASP proteins in more detail.

The biophysical details of the anti-capping and anti-branching mechanisms remain unclear. Specifically, how Ena/VASP proteins interact with the barbed ends of actin filaments and yet allow filament elongation or how Ena/VASP proteins...
inhibit Arp2/3-mediated branching will require detailed structural and kinetic analysis. Furthermore, the relationships among the anti-capping, anti-branching, and Profilin recruitment functions are also unknown. Biochemical and biophysical experiments, possibly involving direct observation techniques such as total internal reflection fluorescence microscopy, will be needed to understand these questions.

Another mystery concerning Ena/VASP proteins is how they are regulated in vivo. All evidence points to PKA/PKG phosphorylation as being a critical regulatory event for mammalian Ena/VASP proteins. It is likely that PKA/PKG phosphorylation regulates Ena/VASP interaction with actin filaments and other binding partners. One challenge will be to understand how Ena/VASP proteins are spatially and temporally phosphorylated within the cell during migration and adhesion. Furthermore, it will be of great interest to understand how tyrosine phosphorylation by c-Abl and protein-protein interactions, such as those involving SH3-Ena/VASP, may regulate Ena/VASP function within cells. Proteomic analysis of the complexes containing Ena/VASP proteins from tissues and cells under different activation conditions will help to unravel the regulation of Ena/VASP proteins. These experiments and others should clarify signal transduction cascades upstream of Ena/VASP proteins and ultimately lead to a greater understanding of how cells regulate the actin cytoskeleton and cell motility.

ACKNOWLEDGMENTS

We thank Geraldine Strasser for the dorsal root ganglion growth cone images appearing in Figure 2.

The Annual Review of Cell and Developmental Biology is online at http://cellbio.annualreviews.org

LITERATURE CITED


Callebaut I, Cossart P, Dehoux P. 1998. EVH1/WH1 domains of VASP and WASP proteins belong to a large family including Ran-binding domains of the RanBP1 family. FEBS Lett. 441:181–85
Domann E, Wehland J, Rohde M, Pistor S,


Halbrugge M, Friedrich C, Eigenthaler M,


Laurent V, Loisel TP, Harbeck B, Wehman A, Grobe L, et al. 1999. Role of proteins of...


Penninger JM, Crabtree GR. 1999. The actin cytoskeleton and lymphocyte activation. *Cell* 96:9–12


Rottner K, Behrendt B, Small JV, Wehland J.
Migfilin and mig-2 link focal adhesions to filament and the actin cytoskeleton and function in cell shape modulation. *Cell* 113:37–47


Figure 1 Expression of Mena in a cultured fibroblast. A fibroblast expressing EGFP-Mena was fixed and labeled with an antibody to Vinculin and with phalloidin (to label actin filaments). Note the enrichment of Mena at the periphery of the lamellipodia, beyond the F-actin labeling, and in adhesions, which label with anti-Vinculin antibody.
Figure 2  Expression of Mena in a cultured dorsal root ganglion (DRG) growth cone and a hippocampal growth cone. Mena strongly labels the distal ends of F-actin bundles (phalloidin stain) in both the DRG and hippocampal growth cones. Anti-Mena labeling also marks the ends of actin-rich extensions in the transition region of both growth cones.
Ena/VASP phosphorylation. Cartoons of the six best-characterized members of the Ena/VASP family are depicted (not to scale). All share a similar domain structure consisting of a central proline-rich region flanked by an amino-terminal EVH1 domain and a carboxy-terminal EVH2 domain. Whereas Ena/VASP proteins are similar in overall structure, in vitro biochemical and in vivo functional data suggest that orthologs from different species have distinct phosphorylation patterns. Although no phosphorylation events have been documented for DdVASP or Unc-34, both have a conserved serine just after the EVH1 domain (comparable to Ser-157 of VASP); the conserved serine in Unc-34 is part of a consensus PKA phosphorylation site (R-R-X-S). Drosophila Enabled does not share this site, but is tyrosine-phosphorylated in vitro and in vivo (Gertler et al. 1995, Comer et al. 1998). Mammalian Ena/VASP proteins all share an amino-terminal PKA/PKG phosphorylation site (Ser-157 of VASP) that, at least in Mena, affects the regulation of cell motility. A neuronal isoform of Mena (not depicted here) is an in vivo substrate for tyrosine kinase activity (Gertler et al. 1996). Recently, more ubiquitous isoforms of Mena have been found to be tyrosine-phosphorylated in vitro (Tani et al. 2003).
CONTENTS

ADULT STEM CELL PLASTICITY: FACT OR ARTIFACT, Martin Raff 1
CYCLIC NUCLEOTIDE-GATED ION CHANNELS, Kimberly Matulef and William N. Zagotta 23
ANTHRAX TOXIN, R. John Collier and John A.T. Young 45
GENES, SIGNALS, AND LINEAGES IN PANCREAS DEVELOPMENT, L. Charles Murtaugh and Douglas A. Melton 71
REGULATION OF MAP KINASE SIGNALING MODULES BY SCAFFOLD PROTEINS IN MAMMALS, Deborah Morrison and Roger J. Davis 91
FLOWER DEVELOPMENT: INITIATION, DIFFERENTIATION, AND DIVERSIFICATION, Moriyah Zik and Vivian F. Irish 119
REGULATION OF MEMBRANE PROTEIN TRANSPORT BY UBQUITIN AND UBQUITIN-BINDING PROTEINS, Linda Hicke and Rebecca Dunn 141
POSITIONAL CONTROL OF CELL FATE THROUGH JOINT INTEGRIN/RECEPTOR PROTEIN KINASE SIGNALING, Filippo G. Giancotti and Guido Tarone 173
CADHERINS AS MODULATORS OF CELLULAR PHENOTYPE, Margaret J. Wheelock and Keith R. Johnson 207
GENOMIC IMPRINTING: INTRICACIES OF EPIGENETIC REGULATION IN CLUSTERS, Raluca I. Verona, Mellissa R.W. Mann, and Marisa S. Bartolomei 237
THE COP9 SIGNALOSOME, Ning Wei and Xing Wang Deng 261
ACTIN ASSEMBLY AND ENDOCYTOSIS: FROM YEAST TO MAMMALS, Åsa E.Y. Engqvist-Goldstein and David G. Drubin 287
TRANSPORT PROTEIN TRAFFICKING IN POLARIZED CELLS, Theodore R. Muth and Michael J. Caplan 333
MODULATION OF NOTCH SIGNALING DURING SOMITOGESIS, Gerry Weinmaster and Chris Kintner 367
TETRASPANIN PROTEINS MEDIATE CELLULAR PENETRATION, INVASION, AND FUSION EVENTS AND DEFINE A NOVEL TYPE OF MEMBRANE MICRODOMAIN, Martin E. Hemler 397
INTRAFLAGELLAR TRANSPORT, Jonathan M. Scholey 423
CONTENTS

THE DYNAMIC AND MOTILE PROPERTIES OF INTERMEDIATE FILAMENTS, 
Brian T. Helfand, Lynne Chang, and Robert D. Goldman 445

PIGMENT CELLS: A MODEL FOR THE STUDY OF ORGANELLE TRANSPORT, 
Alexandra A. Nascimento, Joseph T. Roland, and Vladimir I. Gelfand 469

SNARE PROTEIN STRUCTURE AND FUNCTION, Daniel Ungar and 
Frederick M. Hughson 493

STRUCTURE, FUNCTION, AND REGULATION OF BUDDING YEAST 
KINETOCHORES, Andrew D. McAinsh, Jessica D. Tytell, and Peter K. Sorger 519

ENA/VASP PROTEINS: REGULATORS OF THE ACTIN CYTOSKELETON AND 
CELL MIGRATION, Matthias Krause, Erik W. Dent, James E. Bear, 
Joseph J. Loureiro, and Frank B. Gertler 541

PROTEOLYSIS IN BACTERIAL REGULATORY CIRCUITS, Susan Gottesman 565

NODAL SIGNALING IN VERTEBRATE DEVELOPMENT, Alexander F. Schier 589

BRANCHING MORPHOGENESIS OF THE DROSOPHILA TRACHEAL SYSTEM, 
Amin Ghabrial, Stefan Luschnig, Mark M. Metzstein, and 
Mark A. Krasnow 623

QUALITY CONTROL AND PROTEIN FOLDING IN THE SECRETORY PATHWAY, 
E. Sergio Trombetta and Armando J. Parodi 649

ADHESION-DEPENDENT CELL MECHANOSENSITIVITY, 
Alexander D. Bershadsky, Nathalie Q. Balaban, and Benjamin Geiger 677

PLASMA MEMBRANE DISRUPTION: REPAIR, PREVENTION, ADAPTATION, 
Paul L. McNeil and Richard A. Steinhardt 697

INDEXES

Subject Index 733
Cumulative Index of Contributing Authors, Volumes 15–19 765
Cumulative Index of Chapter Titles, Volumes 15–19 768

ERRATA

An online log of corrections to Annual Review of Cell and Developmental 
Biology chapters (if any, 1997 to the present) may be found at 
http://cellbio.annualreviews.org