The Ena/VASP enigma

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Summary

Ena/VASP proteins are actin-binding proteins that localize to actin stress fibres, the tips of filopodia and the lamellipodial leading edge. In the past few years, a number of seemingly conflicting studies have confused the Ena/VASP field, pointing to roles for these proteins in both promotion and inhibition of actin-dependent processes. Recent discoveries resolve these contradictions and suggest a novel mechanism of Ena/VASP function, in which the proteins function as 'anti-capping' proteins that antagonize capping proteins at the barbed end of actin filaments.

Key words: Ena/VASP, VASP, Mena, Capping protein, Actin, Cell motility, Lamellipodia

Introduction

The mechanisms underlying actin-based cell motility have been the focus of great interest in the cell biology community. A number of key actin regulatory proteins and complexes have been identified. Along with the Arp2/3 complex, the Ena/VASP protein family first attracted wide attention for its role in promoting the actin-driven motility of the bacterial pathogen *Listeria monocytogenes*. In the past three years a number of studies on Ena/VASP proteins have appeared to arrive at contradictory conclusions as to the function of these proteins in actin dynamics and cell motility. Here, we revisit these contradictory results in light of recent observations.

The Ena/VASP family

The sole member of the Ena/VASP family in *Drosophila*, *Enabled* (Ena), was identified through its genetic interactions with the Abl tyrosine kinase and later found to function in several signaling pathways essential for axon guidance in the developing nervous system (Bashaw et al., 2000; Gertler et al., 1995; Gertler et al., 1990; Wills et al., 1999). Vertebrates have three Ena-related genes: *Mena*, *EVL* and *VASP*. VASP was identified as a major protein kinase A (PKA) substrate in platelets (Halbrugge et al., 1990) and subsequently found to localize to focal adhesions, actin stress fibers, the lamellipodial leading edge and to filopodial tips as do Mena and EVL (Gertler et al., 1996; Lanier et al., 1999; Reinhard et al., 1992; Rottner et al., 1999).

Members of the Ena/VASP family share a conserved domain structure consisting of an N-terminal Ena-VASP-homology-1 (EVH1) domain, a central polyproline-rich core and a C-terminal EVH2 domain (Fig. 1). The EVH1 domain binds to proteins that contain motifs with the consensus (D/E)FPPPPX(D/E)(D/E). Subcellular targeting of Ena/VASP proteins to focal adhesions depends upon EVH1-mediated interactions with zyxin and vinculin, both of which contain EVH1-binding sites (Gertler et al., 1996; Niebuhr et al., 1997). Another EVH1 ligand, FYB/SLAP, recruits Ena/VASP to immunological synapses in T-cells and to phagocytic cups in macrophages (Coppolino et al., 2001; Krause et al., 2000). *Listeria* recruits Ena/VASP by displaying the bacterial ActA protein on its surface, which harbors multiple copies of the EVH1 ligand in a classic example of evolutionary mimicry (Chakraborty et al., 1995). The central proline-rich domain contains binding sites for SH3 and WW domains as well as for the G-actin binding protein profilin. The EVH2 domain is required for multimerization and for F-actin binding (Ahern-Djamali et al., 1998; Bachmann et al., 1999; Carl et al., 1999; Laurent et al., 1999).

What is the controversy about Ena/VASP proteins?

Several studies have proposed functions for Ena/VASP proteins that seem to be contradictory. In some cases, Ena/VASP proteins were proposed to promote actin-dependent processes, whereas others suggested an inhibitory role for Ena/VASP in actin-dependent motility. The following results were interpreted as indications of a positive role for Ena/VASP proteins in actin-dependent processes. First, deletion of the Ena/VASP-binding sites within the bacterial protein ActA led to a decrease in actin-dependent intracellular motility of *Listeria monocytogenes* (Niebuhr et al., 1997; Smith et al., 1996). Furthermore, addition of VASP resulted in an increase in bacterial speed in in vitro reconstitution assays of *Listeria* motility (Laurent et al., 1999; Loisel et al., 1999) but was not required for *Shigella* motility (Egile et al., 1999). Second, the amount of GFP-VASP at the tip of lamellipodia correlates with the protrusion velocity of these lamellipodia in B16-F1 mouse melanoma cells (Rottner et al., 1999). Third, delocalization of all Ena/VASP proteins in Jurkat T-cells abolished reorganization of the actin cytoskeleton and therefore polarization of T-cells towards anti-CD3-coated latex beads mimicking antigen-presenting cells (Krause et al., 2000). Fourth, upon delocalization of all Ena/VASP proteins within macrophages, the actin-dependent process of phagocytosis was impaired (Coppolino et al., 2001). Finally, purified Ena/VASP proteins can stimulate the nucleation of actin filaments in vitro (Bachmann et al., 1999; Harbeck et al., 2000; Lambrechts et al., 2000; Laurent et al., 1999).

By contrast, the following studies were interpreted as

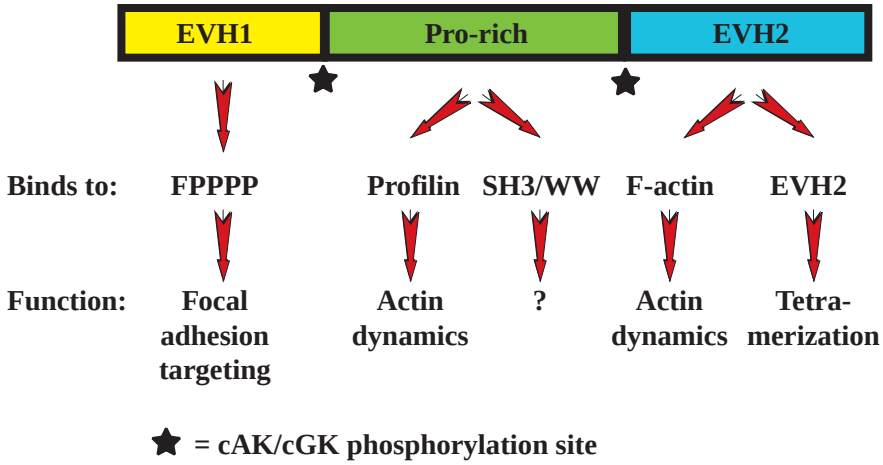

Fig. 1. Domain structure of Ena/VASP family proteins. The domain structure of the Ena/VASP proteins along with their binding partners and functions are shown.

indications of a negative role for Ena/VASP proteins in actin-dependent cellular processes. First, platelets isolated from VASP knockout mice exhibit increased rates of collagen-induced platelet aggregation (an actin-dependent process) compared with wild-type platelets (Aszodi et al., 1999; Hauser et al., 1999). Second, fibroblasts devoid of Ena/VASP proteins exhibit increased rates of cell motility (Bear et al., 2000). Third, Bashaw and colleagues presented genetic evidence that Ena is required, in part, for the repulsive phenotype of the axon guidance receptor Robo in *Drosophila* (Bashaw et al., 2000). Fourth, neutralization of Ena/VASP function in neurons within the developing neocortex caused the neurons to migrate significantly farther than normal neurons. The aberrant superficial placement of Ena/VASP-inhibited neurons was cell autonomous, and the phenotype is consistent with increased rates of neuronal migration (Goh et al., 2002).

How can these apparent discrepancies be explained?

It is important to consider the processes that were analyzed in the aforementioned studies. On the one hand, activities of whole cells or cell behavior in the context of whole animals were examined (fibroblast migration, platelet aggregation, cortical neuron migration or axon guidance). On the other hand, the behavior of parts of cells (the speed of individual lamellipodia, the formation of F-actin-dependent membrane protrusions of activated T-cells, the formation of phagocytic cups or movement of bacteria within cells or in vitro) were characterized.

Although all these processes are actin dependent, they are not directly comparable. A protein that positively regulates actin polymerization might change the geometry of the actin filament network in addition to stimulating actin assembly. The net effect of such changes in the filamentous actin network may not be easy to predict. The notion that the amount of actin polymerization is positively correlated with cell motility rates is not necessarily true. For example, although it is widely accepted that capping protein blocks monomer addition at the barbed ends of actin filaments, overexpression of capping protein in *Dictyostelium* results in faster cell migration (Hug et al., 1995). Therefore, here we first discuss the function of Ena/VASP proteins in actin polymerization at a molecular level and then explain the outcomes of the different approaches used to study Ena/VASP function in cells and organisms.

What is the effect of Ena/VASP proteins on actin polymerization?

Some reports have described VASP as a direct nucleator of F-actin. But in these reports purified VASP nucleates actin polymerization only at low, non-physiological salt concentration (Bachmann et al., 1999; Harbeck et al., 2000; Laurent et al., 1999). Nevertheless, EVL can reduce the lag phase in in vitro actin polymerization assays at physiological salt concentrations (Lambrechts et al., 2000). Are Ena/VASP proteins then nucleators of actin filaments in living cells? Targeted, subcellular sequestration of Ena/VASP proteins to the surface of mitochondria (where they are not normally enriched) provided a way to test this hypothesis in living cells: Ena/VASP proteins were recruited to mitochondria presenting these Ena/VASP-binding sites but F-actin co-recruitment could not be detected. Furthermore, *Listeria* that exhibit defective Arp2/3 recruitment but still recruit normal levels of Ena/VASP protein fail to assemble or recruit any detectable F-actin (Bear et al., 2000; Lasa et al., 1997; Pistor et al., 1994; Pistor et al., 1995; Pistor et al., 2000; Skoble et al., 2000). One recent report, however, showed recruitment of VASP and F-actin to mitochondria of cells transfected with the Ena/VASP binding sites of zyxin fused to a mitochondrial membrane anchor (Fradelizi et al., 2001). One potential reason for this contradiction is that Fradelizi et al. used saponin-permeabilized cells and added exogenous G-actin in their assay, whereas the reports that failed to observe Ena/VASP-dependent (and Arp2/3-independent) F-actin assembly/recruitment used live, intact cells. The inconsistent results might have arisen because permeabilization changes the composition and concentration of cytosolic molecules with respect to actin and therefore may not reflect physiological conditions. Therefore, we conclude that there is no persuasive evidence that supports a role for Ena/VASP proteins as nucleators of actin within living cells.

So how do Ena/VASP proteins regulate actin polymerization? One clue comes from their localization: they are concentrated in places that contain growing free barbed ends of actin filaments, such as the distal tips of lamellipodia or filopodia. In fact, the distribution of Ena/VASP to these

structures depends upon their interaction with growing barbed ends. Treatment of cells with low doses of cytochalasin D, a drug that binds barbed ends and blocks polymerization, delocalizes Ena/VASP proteins from the tips of lamellipodia and filopodia (Bear et al., 2002). This observation is in agreement with earlier observations that VASP binds in vitro to F-actin (Bachmann et al., 1999; Laurent et al., 1999) but clearly demonstrates that this binding is distinct from that of other proteins that bind along the sides of actin stress fibers (reviewed in Pollard and Cooper, 1986). Finally we reported that Ena/VASP proteins antagonize the ability of capping protein to inhibit actin polymerization at barbed ends in vitro (Bear et al., 2002). These results led us to propose that Ena/VASP proteins associate with actin filaments at or near the barbed end and protect them from being capped by capping protein.

This proposed function for Ena/VASP is consistent with the observed alterations of actin geometry within the lamellipodia of cells that either contain elevated levels of or lack Ena/VASP proteins. Ena/VASP-deficient lamellipodia contain networks of shorter, more highly branched actin filaments compared with control cells. By contrast, cells in which all Ena/VASP proteins are overexpressed or constitutively targeted to the membrane contain networks of longer, less branched actin filaments (Bear et al., 2002). Therefore, Ena/VASP proteins appear to support F-actin assembly within cells by acting as 'anti-capping' proteins. Ena/VASP activity also appears to reduce filament branching. Whether this effect is due to suppression of Arp2/3 function or a decrease in branch stability remains to be determined, although it is unlikely that Ena/VASP proteins bind to the Arp2/3 complex.

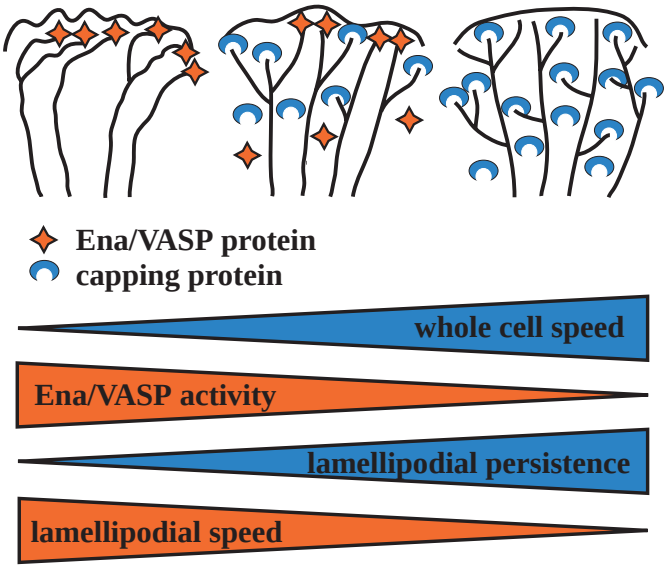

Fig. 2. Antagonism between Ena/VASP proteins and capping protein regulates lamellipodial protrusion and whole cell motility. Elevated Ena/VASP activity at the leading edge antagonizes capping protein activity, resulting in longer less branched filaments. This leads to higher lamellipodial protrusion velocity but decreased persistence. Conversely, reduced Ena/VASP activity at the leading edge results in shorter, more highly branched networks of actin filaments. This results in higher lamellipodial persistence and leads to faster whole cell migration speeds despite decreased lamellipodial protrusion velocity.

How does the effect of Ena/VASP proteins on actin polymerization lead to differences in whole cell behavior?

Lamellipodial protrusion is a key step in cell movement. Superficially, it seems reasonable to presume that the instantaneous velocity of lamellipodial protrusion should correlate in some manner with the overall rate of cell movement; such a relationship, however, has never been demonstrated. In fact, careful analysis of individual lamellipodia devoid of all Ena/VASP proteins revealed that such lamellipodia protrude slower than controls but persist significantly longer. Since net motility is an integration of individual component steps over time, the slow but persistent protrusion of Ena/VASP-deficient lamellipodia provides an explanation as to why cells move faster in the absence of Ena/VASP function. By contrast, fibroblasts in which all Ena/VASP proteins were targeted to the plasma membrane exhibited rapid lamellipodial protrusions that withdrew quickly (Bear et al., 2002), leading to the observed decrease in whole cell movement. This observation is consistent with previous reports that the amount of VASP at the tip of an individual lamellipodium correlates with its velocity (Rottner et al., 1999). The apparent paradox between Ena/VASP localization in protruding lamellipodia and their negative effect on whole cell speed can be explained by the fact that whole cell speed correlates with the persistence of lamellipodial protrusion rather than the velocity of individual protrusions. At a mechanistic level, Ena/VASP influences the fate of individual protrusions by regulating the geometry of the actin cytoskeleton at the leading edge. Although Ena/VASP can control both filament length and branching density, experiments with low doses of cytochalasin D (which selectively affects length and not branching) suggest that the effects on filament length are sufficient to explain the observed Ena/VASP-dependent changes in lamellipodial dynamics. A similar mechanism may explain why *Dictyostelium* cells with elevated levels of capping protein move faster than controls, although lamellipodial dynamics and actin geometry were not quantified in this study (Hug et al., 1995).

What is the potential significance of Ena/VASP influencing cell motility rates by regulating barbed end elongation? Protruding lamellipodia contain a high density of barbed ends, which in turn could promote recruitment of Ena/VASP proteins. At one extreme, excess Ena/VASP anti-capping activity would create a network of long actin filaments that would presumably be too flexible to counteract the forces of membrane tension. At the other extreme, the absence of Ena/VASP activity would favor the formation of networks of short and therefore stiff filaments that permit protrusions to persist longer and potentially increase the probability that they are stabilized further by the formation of cell substratum attachments (Fig. 2).

The activity of Ena/VASP proteins is regulated by phosphorylation, and members of this family appear to interact with a variety of signaling molecules (Gertler et al., 1996; Halbrugge et al., 1990; Loureiro et al., 2002). Therefore, Ena/VASP may integrate multiple signaling pathways responding to the extracellular environment at the very tip of

protruding lamellipodia. Signals that increase Ena/VASP activity would promote the formation of ruffles, thereby increasing the membrane surface area while causing cells to slow down. Such a mechanism might permit cells to explore the environment and to regulate their 'motility machinery' accordingly.

This might also help to explain the different results that were obtained in T-cells and macrophages. Membrane extensions are formed during T-cell activation and phagocytosis but the actin ultrastructure of these protrusions is less well studied than that of lamellipodia of fibroblasts. Perhaps, these membrane extensions resemble ruffles more than lamellipodia. Any actin-based process that depends on relatively long and unbranched actin filaments would probably be positively influenced by Ena/VASP activity. Neuronal growth cones, which extend many long filopodia composed of bundled, unbranched actin filaments, may be particularly dependent on Ena/VASP activity.

How can we reconcile the results of studies on platelets that were interpreted to indicate a negative regulatory function of Ena/VASP proteins on the actin cytoskeleton? Platelet activation and aggregation is a complex process involving the formation of filopodia, lamellipodia and finally contraction of the platelets, each of which may depend upon Ena/VASP function. Since only the total rate of aggregation was measured, the effect of the absence of VASP on the speed of lamellipodia and filopodia protrusions might be obscured. Analysis of the actin ultrastructure of the processes of phagocytosis, T-cell polarization, platelet aggregation and growth cone guidance might help to answer this question.

How are Ena/VASP proteins recruited to the tips of lamellipodia and filopodia?

As noted above, targeting of Ena/VASP proteins to the tips of lamellipodia and filopodia requires their interaction with the barbed ends of actin filaments. This interaction alone, however, is not sufficient for proper targeting to lamellipodial tips. The C-terminal EVH2 domain contains all of the known actin-binding motifs within Ena/VASP proteins. Nevertheless, an isolated EVH2 domain fused to GFP results in GFP targeting to a broad region of the lamellipodia (and not to focal adhesions) in Ena/VASP-deficient cells (Bear et al., 2002; Loureiro et al., 2002). Nakagawa and colleagues have also studied the role of the EVH2 domain in subcellular targeting of Ena/VASP proteins. They noted that the EVH2 domain is sufficient to direct GFP concentration to the edges of spreading cells. Their interpretations, however, are complicated by the presence of endogenous wild-type Ena/VASP protein in the cell types used in their study because a coiled-coil motif within the EVH2 domain mediates oligomerization with endogenous Ena/VASP proteins (Nakagawa et al., 2001). A second complication of this study is that Nakagawa and colleagues analyzed the edges of cells plated using a protocol requiring 16 hours for full cell spreading rather than examining lamellipodia of motile cells.

The EVH1 domain might act to refine the distribution of Ena/VASP within lamellipodia to the very tip. Although other EVH1-binding proteins such as zyxin do not localize to lamellipodia (Rottner et al., 2001), we have recently identified a novel EVH1-binding protein that is concentrated in lamellipodial and filopodial tips and therefore could participate in anchoring and/or regulating Ena/VASP activity at the tips of these structures (M.K. and F.G., unpublished).

What is the significance of profilin binding to Ena/VASP?

Originally, models for Ena/VASP function focused on their ability to bind profilin through their proline-rich core. Profilin is an abundant G-actin-binding protein that participates in loading actin monomers with ATP. When bound to profilin, actin monomers are preferentially added onto the barbed ends of actin filaments (reviewed in Schluter et al., 1997; Sun et al., 1995). Now that Ena/VASP proteins have been assigned an 'anticapping' function, it is worth considering the role of profilin binding in Ena/VASP function.

First, the anticapping activity appears to be sufficient for Ena/VASP function in regulating random fibroblast migration and lamellipodial dynamics. Deletion of the polyproline-rich region of Mena, which contains the profilin-binding sites, has no effect on the ability of Mena to rescue the hypermotile phenotype of Ena/VASP deficient cells (Loureiro et al., 2002). What then is the potential function of Ena/VASP-profilin complexes? Unlike lamellipodial dynamics, *Listeria* motility appears to require the polyproline region of Ena/VASP proteins for optimal intracellular motility (Geese et al., 2002). Since profilin recruitment to the bacterial surface correlates with motility rates, it is possible that Ena/VASP proteins act as adaptors to recruit profilin to the bacterial surface (Geese et al., 2000).

Skoble and colleagues have noted that *Listeria* expressing a mutant ActA protein lacking the G-actin binding site require Ena/VASP to support Arp2/3-dependent actin nucleation on the bacterial surface (Skoble et al., 2001). It is possible that Ena/VASP recruits profilin to supply polymerization-competent monomers to support Arp2/3-dependent nucleation on the bacterial surface, although the high intracellular concentration of profilin (20 μM) makes it unclear why such a mechanism would be necessary (for reviews, see Schluter et al., 1997; Sun et al., 1995). Interestingly, *Listeria* apparently do not utilize the anticapping activity of Ena/VASP proteins because deletion of motifs essential for this activity has no effect on the ability of the proteins to support *Listeria* motility but does eliminate their function within lamellipodia (Geese et al., 2002; Loureiro et al., 2002).

It is unclear which physiological processes use the Ena/VASP-profilin interaction in addition to, or instead of, the anticapping activity of these proteins. The affinity of Ena/VASP proteins for profilin is in the nanomolar range, and the polyproline-binding activity of profilin is required for profilin function, at least in yeast (Lambrechts et al., 2000; Lu and Pollard, 2001). The phenotype of *Mena* knockout mice is exacerbated by a reduction in the gene dosage of profilin from two to one (Lanier et al., 1999). Although this type of genetic evidence does not prove that Ena/VASP proteins and profilin function in a biochemical complex, it does indicate that Mena and profilin participate in some overlapping pathways. Besides random fibroblast migration there are other cellular actin-dependent processes, such as filopodia dynamics, phagocytosis, axon guidance or vesicle movement that might require recruitment of profilin through Ena/VASP proteins.

Further analysis will be required to identify cellular processes that depend upon Ena/VASP-profilin complexes.

Concluding Remarks

Although a good deal of the confusion about the function of Ena/VASP proteins has been resolved by recent studies, a number of outstanding areas remain to be investigated. Mena has been identified in complexes containing a number of SH3- and WW-domain adaptors such as FE65 and IRSp53 (Ermekova et al., 1997; Krugmann et al., 2001). In light of the fact that a deletion variant of Mena lacking the binding sites for these molecules (Pro-rich deletion) can fully rescue fibroblast motility, there is no evidence that such interactions are required for basic fibroblast motility. However, other actin-dependent processes involving Ena/VASP proteins may have different requirements for co-factors such as these adaptor proteins and their binding partners. Future studies will be required to elucidate the role of Ena/VASP in these processes.

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