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ENA/VASP PROTEINS: MULTIFUNCTIONAL REGULATORS OF ACTIN CYTOSKELETON DYNAMICS

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1. ABSTRACT

The spatial and temporal regulation of the actin cytoskeleton is fundamental to several cellular processes as diverse as cell motility and immune responses. At the molecular level, the remodelling of the actin cytoskeleton depends on two key events: actin filament nucleation and elongation. Seminal studies on the actin-based intracellular motility of the bacterial pathogen *Listeria monocytogenes* have been instrumental for the characterisation of a class of actin filament elongating factors, the proteins of the Ena/VASP family. Ena/VASP proteins enhance actin filament elongation via the recruitment of profilin:actin complexes to sites of active actin remodelling such as the tips of spreading lamellipodia and the surface of intracellular *Listeria*. Moreover, Ena/VASP proteins not only enhance actin filament elongation but also influence the activity of the Arp2/3 complex and counteract the inhibition of actin polymerisation by capping proteins. These findings, taken together with the observation that Ena/VASP proteins can influence actin filament architecture by affecting the actin filament branching activity of the Arp2/3 complex, define Ena/VASP proteins as multifunctional organisers of the actin cytoskeleton.

2. INTRODUCTION

Several cellular activities such as cell division, embryonic development, axonal pathfinding and some types of immune responses are all linked to the co-ordinated remodelling of the actin cytoskeleton. The spatial and temporal assembly of actin filaments is regulated by a plethora of proteins associated with the actin cytoskeleton that control one or more of the activities that shape this important cellular structure: actin filament nucleation, branching, capping and severing as well as actin monomer sequestration and bundling of actin filaments. In recent years, several investigations have provided compelling evidence that an essential step toward the remodelling of the actin cytoskeleton is the *de novo* nucleation of actin filaments. This process is mainly controlled by the activity of the Arp2/3 complex, a multiprotein complex that is composed of two actin-related proteins Arp2 and Arp3 and five smaller proteins that in the human Arp2/3 complex are referred to as p41-Arc, p34-Arc, p21-Arc, p20-Arc and p16-Arc (1, 2). The Arp2/3 complex does not regulate actin filament nucleation (and branching) on its own but it requires the interaction with actin filaments and several adapter proteins such as those of the Scar/Wave and WASP/N-WASP family for optimally performing as nucleator of actin filaments (see 3, 4). Because actin filament nucleation *per se* is not sufficient to sustain the fast elongation of actin filaments that drives, for example, the rapid advancement of lamellipodia other cellular factors must be involved in this process. Studies on the actin-driven intracellular motility of the bacterial pathogen *Listeria monocytogenes* were instrumental for the characterisation of these 'filament elongating' factors: the proteins of the Ena/VASP family. Originally discovered as substrates for cAMP- and cGMP-dependent protein kinases in platelets (see 5), this protein family includes the founding member the *Drosophila* protein Ena and three mammalian proteins, the Vasodilator-Stimulated Phosphoprotein (VASP), the mammalian Enabled (Mena), and the Ena-VASP-like (EVL) protein (6-9). In the mouse, the expression of Ena/VASP proteins takes place in many tissues (brain, lung, spleen and stomach) and cell types

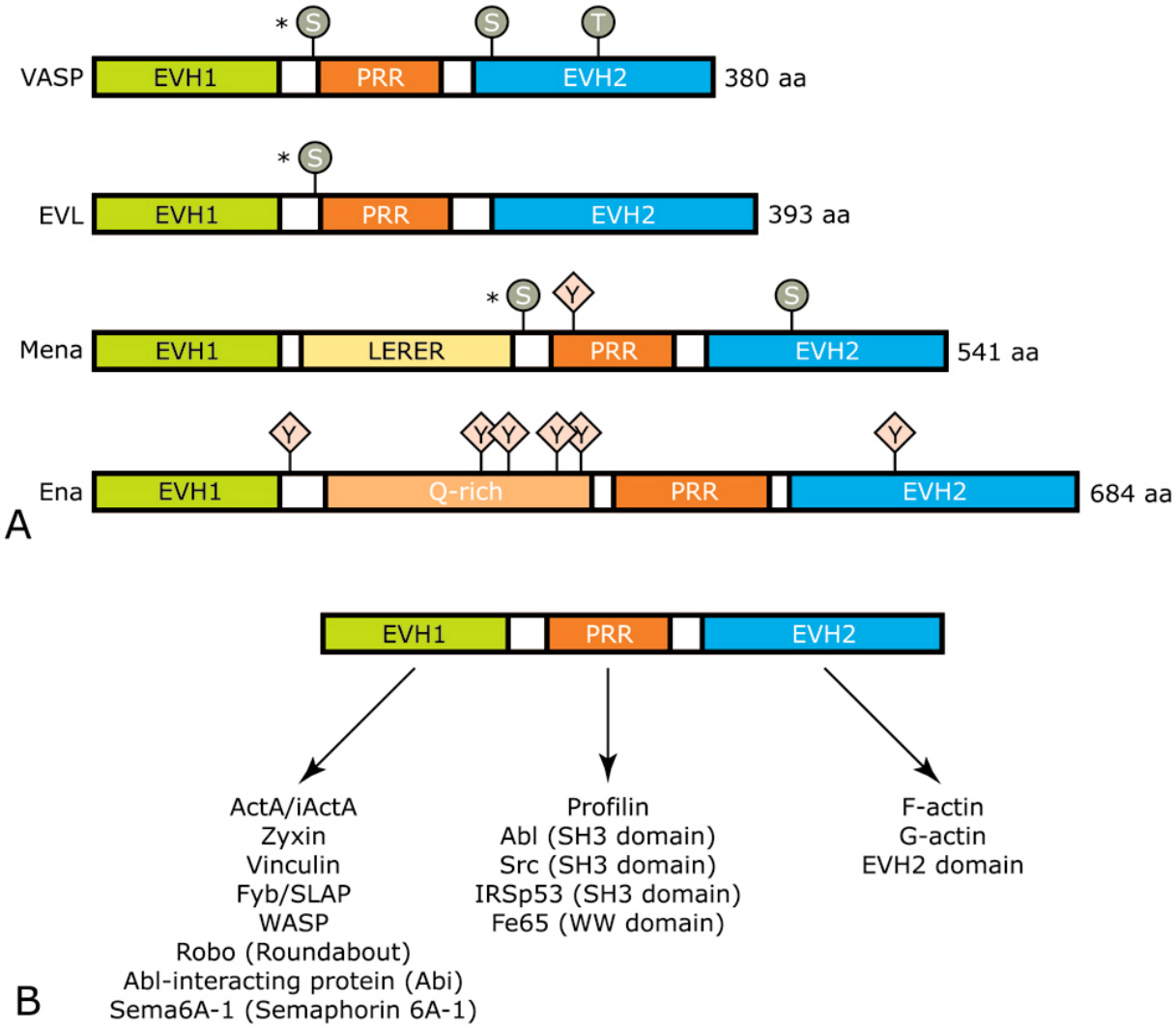

Figure 1. Ena/VASP proteins and their binding partners. (A) Domain organisation of Ena/VASP proteins. EVH1: Ena/VASP Homology 1, PRR: proline-rich region, LERER: region harbouring LERER repeats, Q-rich: glutamine-rich region, EVH2: Ena/VASP Homology 2. Serine and Threonine phosphorylation sites are indicated as dark-green circles. The conserved Ser phosphorylation site is indicated with an asterisk. Tyrosine phosphorylation sites are indicated as pink rhombuses. (B) Major binding partners for the EVH1, EVH2 and PRR regions of Ena/VASP proteins.

(fibroblasts, endothelial and epithelial cells and lymphocytes) and is regulated during the postnatal development (10-12). In this review, we will discuss known and potential functions of Ena/VASP proteins and their implications for our understanding of the regulation of actin cytoskeleton remodelling.

3. DOMAIN STRUCTURE AND BINDING PARTNERS OF ENA/VASP PROTEINS

Ena/VASP proteins share several structural and functional features. At the N-terminus they have a ~100 amino acid domain, the Ena/VASP homology 1 (EVH1) domain, which is highly conserved among all members of this protein family. Their central portion is characterised by a proline-rich region (PRR) that contains multiple copies of the GPPPPP motif. The C-terminus of Ena/VASP proteins, the Ena/VASP homology 2 (EVH2) domain, varies is size from about 150 amino acids in VASP to about 190 amino acids in Ena (figure 1).

The EVH1 domain serves as binding site for proteins which harbour the motif F/LPPPP including the listerial proteins ActA and iActA and the cytoskeletal and adapter proteins vinculin, zyxin, Fyb/SLAP and WASP (13-20) (figure 2). Other proteins that contain the F/LPPPP motif include Shroom, palladin and the proline-rich RNA-binding protein (prrp) (21-23), although in this case a direct association with the EVH1 domain of Ena/VASP proteins has not yet been demonstrated. The central proline-rich region of Ena/VASP proteins mediates the interaction of these proteins with several ligands. Studies based on biochemical assays have led to the identification of the first ligand for this region, the actin

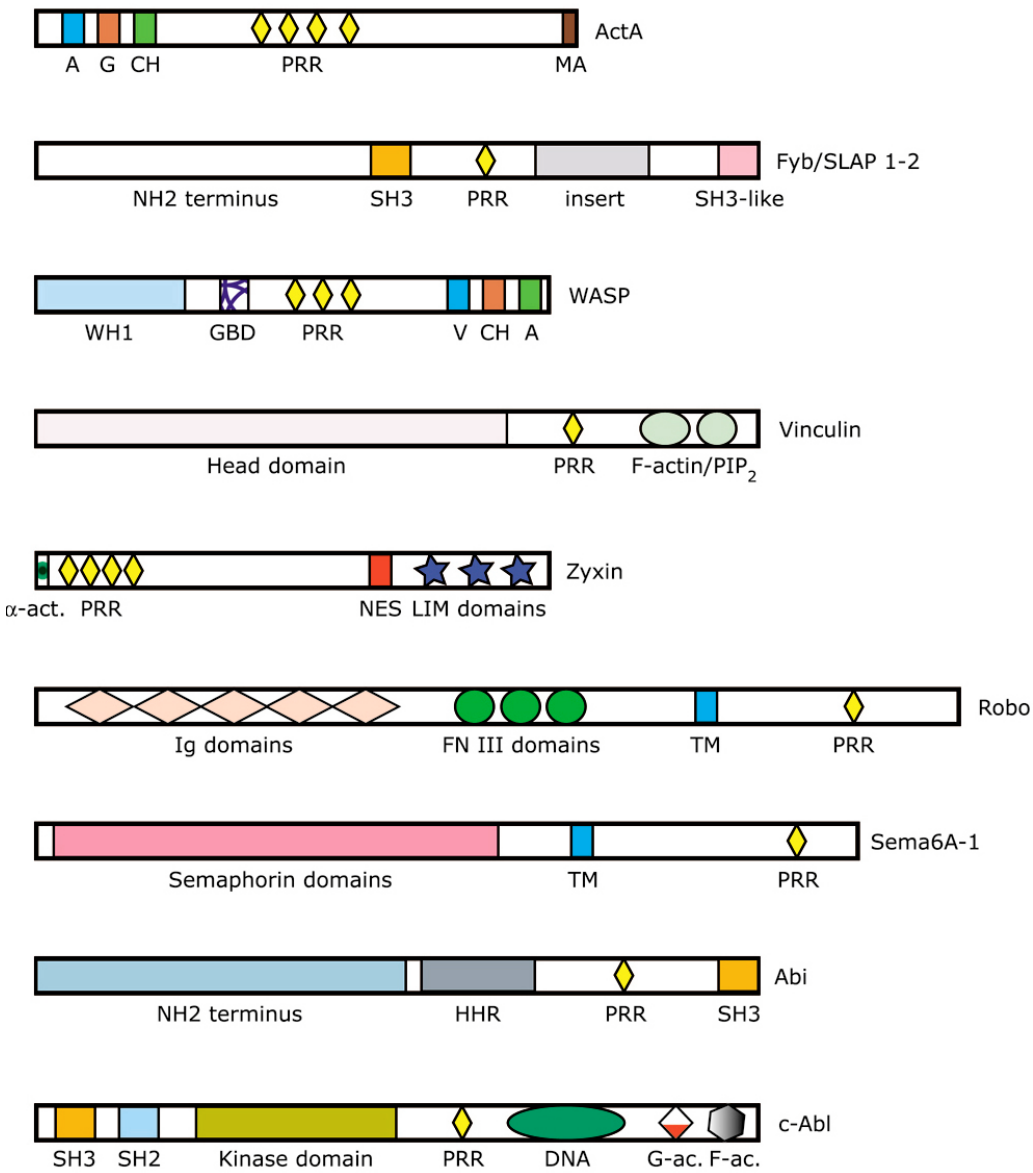

Figure 2. Domain organisation of the best characterised binding partners of Ena/VASP proteins. A: acidic region, G: G-actin-binding region, CH: cofilin homology, MA: membrane anchor, SH3: Src homology 3, WH1: WASP homology 1, GBD: GTPases-binding domain, α-act.: alpha actinin, NES: nuclear export signal, FN III: fibronectin type III, TM: trans-membrane domain, HHR: Homeobox homology region, SH2: Src homology 2, G-ac.: G-actin, F-ac.: F-actin.

monomer-binding protein profilin (24, 25). Later studies have shown that the PRR of Ena/VASP proteins can associate with SH3 and WW domains. In particular, it can interact with the SH3 domain of the non-receptor tyrosine kinases Abl and Src and of the substrate of the insulin receptor tyrosine kinase IRSp53 (7, 26-28) and with the WW domain of the neuronal protein FE65 (29). Lastly, the EVH2 domain of Ena/VASP proteins harbours binding sites for monomeric (G-actin) and filamentous (F-actin) actin and can mediate the multimerisation of Ena/VASP proteins (30-32).

Other than being characterised by the above mentioned domains and binding regions, Ena/VASP proteins contain conserved phosphorylation consensus sequences for the cAMP- and cGMP-dependent serine/threonine protein kinases PKA and PKG (7, 11, 33) and for the protein tyrosine kinase Abl (34; figure 1).

4. *IN VITRO* PROPERTIES OF ENA/VASP PROTEINS

The direct binding of Ena/VASP proteins to F-actin was demonstrated for the first time by Reinhard and colleagues (10), who showed that purified VASP could be recovered in F-actin pellets after high speed centrifugation. This observation was later corroborated by several independent studies (30, 31, 35, 36). Further investigations

indicated that residues 259-276 (in human VASP) within the EVH2 domain are essential for the interaction of Ena/VASP proteins with F-actin (30). One macroscopic consequence of this interaction is the increase in the light scattering and turbidity of F-actin solutions due to the formation of F-actin bundles (30, 31, 35). The ability of Ena/VASP proteins to bundle F-actin may increase the stability of these actin structures as suggested by the observation that these proteins protect actin filaments from the severing effect of gelsolin *in vitro* (37). An interesting feature of the Ena/VASP:F-actin interaction is that it is more efficient under low salt conditions (15-60 mM KCl) suggesting that it primarily depends on electrostatic interactions between F-actin and Ena/VASP proteins (11, 31, 36). The notion that electrical charges mediate this interaction is supported by the finding that PKA- and PKG-dependent phosphorylation of VASP reduces the amount of this protein that co-sediments with F-actin (36). By contrast, an independent study showed that phosphorylated VASP binds indeed better to F-actin (35). As the two studies are not directly comparable (they use different expression systems to purify VASP, different methods to phosphorylate it and buffers having different ionic strength) caution should be exerted in judging these conclusions. However, because actin filaments are negatively charged (38) and the binding of actin filament to fascin is reduced upon fascin phosphorylation (39), it is likely that adding negative charges to Ena/VASP proteins would favour their repulsion from actin filaments, and hence reduce their interaction with them.

While the interaction of Ena/VASP proteins with F-actin *in vitro* causes the bundling of actin filaments, the incubation of these proteins with G-actin leads to actin filament polymerisation (31, 35). This effect of Ena/VASP proteins on actin filament growth is characterised by a reduction in the lag phase of polymerisation and by a faster polymerisation rate (31, 35). Consistent with its ability to bind to F-actin, the EVH2 domain of Ena/VASP proteins is as efficient as full-length proteins in inducing actin filament polymerisation (31, 35, 40). Thus, Ena/VASP proteins may induce actin filament polymerisation by stabilising the actin nuclei (trimers) or by stabilising actin filaments or both.

Since Ena/VASP proteins induce actin polymerisation, they may interact with monomeric actin. The EVH2 domain of Ena/VASP proteins harbours a short positively-charged sequence that highly resembles to the KLKK motif of thymosinβ4 that mediates the interaction of this protein with G-actin (41). A recent study reported that the KLKR motif of Ena/VASP proteins interacts with G-actin as suggested by the observation that the mutation of critical arginine and lysine residues within this motif abolishes its binding to G-actin (32). Curiously, a VASP mutant lacking the multimerisation site but harbouring an intact KLKR motif failed to bind to G-actin in this assay. On the other hand, this VASP mutant and VASP proteins carrying mutations in this motif can interact with G-actin after longer incubation times (32). Moreover, VASP proteins harbouring mutated KLKR motifs are not able to induce actin polymerisation *in vitro* although they have an intact F-actin-binding site (32). Given that the association of Ena/VASP proteins with F-actin is necessary for their ability to induce actin polymerisation *in vitro* (11) and assuming that the two point mutations introduced in VASP are unlikely to cause gross disturbances in the overall folding of this protein, it is not clear why such VASP mutants have lost their function in this assay. Future studies should be aimed at clarifying these issues.

Although Ena/VASP proteins can induce actin assembly *in vitro*, several observations suggest that they do not so in living cells. When Ena/VASP proteins are constitutively targeted to the surface of mitochondria (cellular organelles where they normally do not localise) no F-actin accumulation can be detected following staining with fluorescent phalloidin. Moreover, *Listeria* mutants expressing an ActA variant that exhibits defective recruitment of the Arp2/3 complex but normally binds to Ena/VASP proteins do not associate with detectable levels of F-actin (42-46). One study, however, reported that the targeting of Ena/VASP proteins to beads coated with the proline-rich region of ActA causes the accumulation of F-actin following their incubation with extracts of cultured cells. Similarly, the zyxin-dependent recruitment of Ena/VASP proteins at the surface of mitochondria also results in the accumulation of F-actin at these structures (47). As the actin polymerisation at the surface of Ena/VASP-coated beads can be inhibited by pre-incubation of the beads with the proline-rich region of ActA, it was concluded that Ena/VASP proteins are responsible for actin polymerisation at the bead surface (47). It should be noted, however, that pre-incubation of the beads with the WA domain of WASP (which is responsible for the stimulation of Arp2/3 complex-dependent actin polymerisation and is therefore expected to block the activity of this complex) significantly impaired actin accumulation on the beads (47) indicating that additional mechanisms were responsible for actin polymerisation at the surface of Ena/VASP-coated beads. Finally, it also should be considered that permeabilisation of cells and incubation with rhodamine-labelled G-actin prior to fixation certainly altered the physiological conditions within the cells so introducing potential artifacts. Thus, at present, there is no convincing evidence for supporting Ena/VASP protein-dependent actin nucleation in living cells.

As elaborated above, the central proline-rich region of Ena/VASP proteins associates with profilin, an actin monomer-binding protein that depending on the status of the actin filament (+)-ends (uncapped or capped) promotes or inhibits actin filament elongation (48-50). Moreover, profilin enhances the release of ADP from actin and synergises with other actin monomer-binding proteins such as thymosinβ4 and the actin depolymerising factor (ADF)/cofilin to increase actin filament turnover (51, 52). In the context of Ena/VASP protein-profilin interaction, these proteins may affect actin polymerisation *in vitro* at yet another level given that a complex formed by profilin (specifically profilin II) and the PRR of VASP is able to overcome the inhibitory effect of thymosinβ4 on, and reduces the lag phase of, actin polymerisation (53). Regardless of the mechanisms underlying this effect, these data suggest that the proline-rich region of Ena/VASP

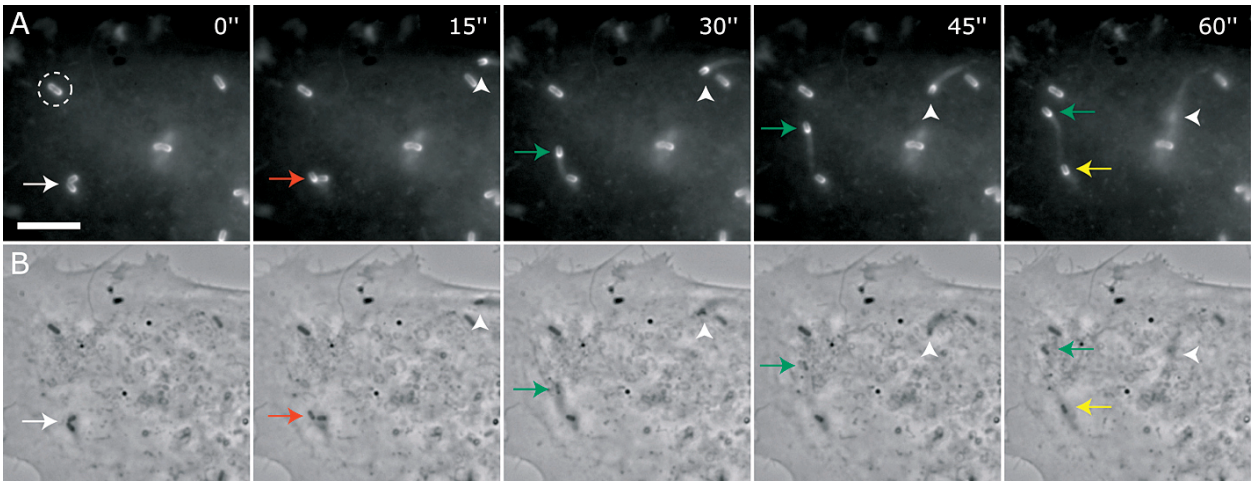

Figure 3. Dynamics of GFP-VASP during *Listeria* motility. Panels in (A) show GFP-VASP, whereas panels in (B) show the corresponding phase contrast images. GFP-VASP usually localises in a uniform manner at the surface of non-motile bacteria (dashed circle). Following a bacterial division (white arrow), *Listeria* acquire the ability to move and concomitantly with the onset of bacterial motility the distribution of GFP-VASP is polarised being more concentrated at the interface between bacteria and actin tails (red and yellow arrows). Such localisation of GFP-VASP at the bacterial surface is maintained during bacterial motility (white arrowheads and green arrows). Scale bar represents 5 µm.

proteins not only provides the actin monomers required for actin filament elongation but also may contribute to the release of actin from profilin:actin complexes.

Concerning the interaction of Ena/VASP proteins with G- and F-actin, Skoble *et al*. (54) have shown that purified VASP can rescue the ability of an ActA mutant, which cannot bind to G-actin, to support Arp2/3 complex-dependent actin filament nucleation, *in vitro*. These authors concluded that Ena/VASP proteins stimulate actin filament nucleation by delivering F-actin to the Arp2/3 complex, implying that the F-actin-binding region within the EVH2 domain plays a major role in this process. However, since in this study a VASP mutant lacking the F-actin-binding region was not tested for its ability to rescue Arp2/3 complex-induced actin filament nucleation, it is not possible to exclude *a priori* other potential mechanisms. Moreover, with respect to the binding of Ena/VASP proteins to G-actin and based on the finding that Ena/VASP mutants lacking the G-actin-binding region do not support efficient actin filament formation at the *Listeria* surface (55), it is conceivable that Ena/VASP-G-actin interactions participate in the rescue of the Arp2/3 complex-induced actin filament nucleation. In the context of the interplay between Ena/VASP proteins and the Arp2/3 complex in regulating actin filament dynamics, it has been shown that VASP reduces the percentage of actin filament branching induced by the Arp2/3 complex (54). By contrast, another study reported no effect of VASP on branching (56). While the discussion of the discrepancies between these two studies is beyond the scope of this article, it is important to point out that VASP indeed reduces the branching frequency of actin filaments, but only when it is bound to ActA-coated beads (57).

Finally, since Ena/VASP proteins can counteract the inhibitory effect of capping proteins on actin filament polymerisation *in vitro* and cannot bind to capped actin filaments, it has been concluded that Ena/VASP proteins interact with actin filaments at or near their barbed ends and function as anti-capping proteins (58). Conversely, a recent study showed that VASP binds equally well to capped and uncapped actin filaments and that it does not inhibit the capping of barbed ends by capping proteins. Moreover, although VASP reverses the inhibition on actin polymerisation by capping protein, it does so via an indirect competition with capping proteins as indicated by the observation that a fixed concentration of VASP is able to enhance the motility of ActA-coated beads in a solution of pure proteins regardless of the concentration of capping protein (57).

Taken together, the above studies establish that Ena/VASP proteins can affect actin filament dynamics *in vitro* not only by supporting actin polymerisation via the delivery of actin monomers to growing filament (+)-ends, but also by affecting the activities of profilin, the Arp2/3 complex and capping proteins.

5. FUNCTIONS OF ENA/VASP PROTEINS *IN VIVO*

5.1. Intracellular motility of *Listeria monocytogenes*

Studies on the intracellular motility of the bacterium *Listeria monocytogenes* have been instrumental for the understanding of the *in vivo* function(s) of Ena/VASP proteins. Ena/VASP proteins localise at the surface of stationary and motile intracellular bacteria. In the latter case, Ena/VASP proteins are asymmetrically distributed at the interface between bacteria and actin tails (59) (figure 3, Movie 3). The targeting of these proteins to the *Listeria* surface is mediated by the interaction of their EVH1 domains with the central proline-rich region of ActA (15, 60). The importance of this interaction for bacterial motility has been demonstrated through the generation of *Listeria* mutants expressing an ActA protein lacking the proline-rich region. These bacteria did no longer recruit

Ena/VASP proteins and, as a consequence, moved at a slower speed (3-5 times) compared to wild-type bacteria (15, 60).

Since Ena/VASP proteins bind to profilin, which in turn enhances actin filament elongation, it was proposed that one function of Ena/VASP proteins in *Listeria* motility is to deliver profilin:actin complexes to the bacteria surface to support efficient actin filament elongation. Accordingly, it was observed that a *Listeria* mutant unable to recruit Ena/VASP proteins does not recruit profilin too (55, 60, 61). Furthermore, *Listeria* speed directly correlates with the amount of profilin at its surface (61) and the absence of both Ena/VASP proteins and profilin from cell-free systems significantly reduces bacterial speed (62). The essential role of profilin:actin complexes in supporting efficient actin filament elongation at the bacterial surface is further supported by the finding that the injection of cross-linked profilin:actin, which binds to the (+)-ends of actin filament thereby inhibiting their elongation *in vitro* (50), into *Listeria*-infected cells severely impairs bacterial motility (63). It should be pointed out that Ena/VASP proteins do not merely deliver profilin:actin complexes to the bacterial surface but seem to favour the release of actin from such complexes, thus enhancing actin filament elongation at this site (53). Interestingly, Ena/VASP proteins seem to be required for the persistence of the movement of *Listeria* (64) and of ActA-coated beads (57).

The formation of Ena/VASP multimers at the *Listeria* surface was suggested to increase the number of profilin:actin complexes available for actin filament growth and as a consequence to enhance bacterial motility (25). However, Ena/VASP mutants lacking the multimerisation motif are equally efficient in supporting *Listeria* motility as wild-type proteins indicating that the formation of Ena/VASP multimers is dispensable for bacterial motility (55).

The interaction of Ena/VASP proteins with F-actin is also dispensable for *Listeria* motility and, indeed, retards it (55, 64). Ena/VASP:F-actin interactions may be responsible for the tight association between bacteria and actin tails (65) and may play a role in the function of a 'molecular clamp', which has been proposed to control the orientation and positioning of actin filaments abutting the bacterial surface (66). Consistent with this interpretation is the finding that non-dissociable profilin:actin complexes cause the detachment of bacteria from their tails (63). Notably, in Ena/VASP-deficient fibroblasts expressing a Mena mutant lacking the F-actin-binding site *Listeria* tend to change direction more frequently indicating that this site is required for maintaining directional persistence (64). The contribution of Ena/VASP:F-actin interaction to *Listeria* motility appears to be rather complex. Indeed, Ena/VASP proteins can still localise at the surface and along the actin tails of a *Listeria* mutant which expresses a variant of ActA (ActAΔPRR) lacking the central proline-rich repeats (55), suggesting that these proteins can be targeted to *Listeria* (surface and actin tails) in an EVH1-independent manner most likely via their F-actin binding region. In addition, the expression of GFP-Mena in Ena/VASP-deficient cells enhances the speed of this *Listeria* mutant (55), whereas the deletion of the F-actin binding region in Mena impairs intercellular spreading (a process that directly correlates with bacterial motility) of the *Listeria* mutant ActAΔPRR (64). Although the mechanism(s) underlying this EVH1-independent contribution of Ena/VASP proteins to *Listeria* motility remain to be determined, it may be that these proteins may enhance the efficiency of *Listeria* motility by properly orienting the actin filaments toward the bacterial surface.

Ena/VASP proteins regulate *Listeria* motility at yet another level. Skoble and colleagues (54) have shown that VASP can rescue the ability of an ActA mutant lacking the actin monomer-binding site to support Arp2/3 complex-dependent actin filament nucleation. Consistent with these *in vitro* data, a *Listeria* mutant expressing an ActA protein which cannot recruit both actin monomers and Ena/VASP proteins do not nucleate actin filaments at their surface (54), suggesting that Ena/VASP proteins contribute to actin filament nucleation also *in vivo*. Since the deletion of the actin-monomer-binding site within the EVH2 domain of Mena impairs actin accumulation at the *Listeria* surface and bacterial motility (55), Ena/VASP proteins may stimulate actin filament nucleation at the bacterial surface by supplying actin monomers to the Arp2/3 complex. This hypothesis is consistent with the finding that activators of the Arp2/3 complex such as WASP/Scar proteins require binding to G-actin to support Arp2/3 complex-dependent actin filament nucleation, and that Ena/VASP proteins *per se* can enhance *Listeria* motility in cell-free systems in the absence of profilin (62, 67). One cannot exclude another potential function of the actin-monomer-binding site of Ena/VASP proteins, i.e. the delivery of actin monomers to the actin filament (+)-ends abutting the bacterial surface. However, in the absence of the PRR, Ena/VASP proteins do not efficiently support *Listeria* motility (55), implying that their actin-monomer-binding site does not play a role in, or has a little impact on, this process.

Overall, Ena/VASP proteins contribute to *Listeria* motility by enhancing actin filament elongation and Arp2/3 complex-mediated actin filament nucleation, counteracting capping proteins and regulating actin filament architecture.

5.2. Immune receptor signalling

The studies on *Listeria* motility suggest that the main function of Ena/VASP proteins is to support actin filament elongation. Investigations on two types of immune response, T cell activation and phagocytosis, further indicate that this is a major function of this protein family. Both T cell activation and phagocytosis are characterised by the initial activation of protein tyrosine kinases which in turn phosphorylate several adapter proteins such as linker for activation of T cells (LAT), SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) and Fyn-binding protein (Fyb)/SLP-76-associated protein of 130 kDa (SLAP) (Fyb/SLAP). These components, in combination with other adapter proteins, assemble into multi-molecular complexes which relay signals from active immune receptors to downstream cellular functions

including actin cytoskeleton remodelling, gene expression and cytokine production (see 68-70). One major issue in this field concerns the understanding of the molecular mechanisms underlying actin cytoskeleton remodelling. Ena/VASP proteins co-localise with actin at the interface between anti-CD3-coated beads and T cells and at phagocytic cups. In addition, Fyb/SLAP co-localises with Ena/VASP proteins at these subcellular locations and it directly associates with Ena/VASP proteins. Furthermore, the sequestration of Ena/VASP proteins by the overexpression of the central proline-rich region of ActA in T cells and macrophages blocks the targeting of Ena/VASP proteins to the T cell/bead interface and phagocytic cups thus leading to the severe impairment of actin cytoskeleton remodelling at these sites (18, 71). Thus, Ena/VASP proteins are essential for efficient actin filament dynamics during T cell activation and phagocytosis. The function of Ena/VASP proteins in these processes appears to be linked to their Fyb/SLAP-dependent targeting to a multi-molecular complex, which also includes SLP-76, Nck, WASP and the Arp2/3 complex (18, 71). In light of the direct interaction between Ena/VASP proteins and WASP (19), it may also be possible that the targeting of Ena/VASP proteins to this multi-molecular complex is, at least in part, mediated by this association. Notably, the assembly of such multi-molecular complexes may be required for integrating the activities of Ena/VASP proteins and the Arp2/3 complex to generate optimal actin cytoskeleton remodelling. This notion is supported by studies on *Listeria* motility where the ActA-mediated juxtaposition of Ena/VASP protein and the Arp2/3 complex is essential for efficient bacterial motility. In light of the influence of Ena/VASP proteins on the Arp2/3 complex during *Listeria* motility (see above), it will be important to determine whether these proteins also affect, in a similar way, the activity of the Arp2/3 complex during T cell activation and phagocytosis.

5.3. Cell motility

Given the implication of Ena/VASP proteins in so many actin-based events, it is not surprising that these proteins play a role in the most complex of such processes, cell motility. Cell motility is the result of the spatial and temporal co-ordination of four distinct processes: actin-driven extension of protrusions such as lamellipodia and filopodia, stabilisation of these structures by attachment to the substratum via the formation of focal adhesions, translocation of the cell body, and detachment of the rear part of the cell upon disassembly of focal adhesions at this site (see 72). Due to the complexity of cell motility and based on the consideration that it would be difficult to establish an unequivocal protein-function relationship between Ena/VASP proteins and cell motility as a whole, we will discuss in the following sections the function(s) of this protein family in some of the individual steps that underlie this process.

5.3.1. Ena/VASP protein localisation and function in lamellipodia

The extension of lamellipodia is tightly linked to the polymerisation of actin filaments abutting the inner leaflet of the plasma membrane and relies on the co-ordinated actin filament nucleation and elongation (see 73). The first study implicating Ena/VASP proteins in lamellipodia dynamics came from Rottner *et al*. (74), who showed that these proteins localise at the tips of spreading but not retracting lamellipodia in B16F1 melanoma cells (figure 4, Movie 4). Studies on Ena/VASP-deficient fibroblasts provided evidence that the targeting of Ena/VASP proteins to the tips of lamellipodia appears to be controlled at multiple levels. In these cells, the GFP-tagged EVH1 domain alone weakly localises at the tips of lamellipodia (75), whereas the distribution of the EVH2 domain alone corresponds to a broader region of the lamellipodia (58, 76). Thus, both domains contribute to the localisation of Ena/VASP proteins to the lamellipodia with the EVH1 primarily responsible for restricting it to the lamellipodia tips. The function of the EVH2 domain in this context is linked to its ability to interact with F-actin given that in Ena/VASP-deficient cells a Mena mutant lacking the F-actin-binding site weakly localises to lamellipodial tips (58, 76). In addition, low doses of cytochalasin D, a fungal metabolite that binds to actin filament (+)-ends with high affinity (77), reversibly displace Ena/VASP proteins from the leading edge suggesting that these proteins interact with actin filaments at or near their barbed ends (58). Based on these results, it was concluded that Ena/VASP proteins are mainly targeted to the lamellipodia tips via actin-dependent interactions. This conclusion is, however, questioned by the observation that at similar concentrations of cytochalasin D the displacement of Ena/VASP proteins is irreversible. Furthermore, low to moderate concentrations of both cytochalasin B and D that arrest lamellipodia protrusion (a situation where all actin filament (+)-ends are blocked), have been observed to increase the accumulation of VASP at lamellipodia tips, thus arguing against the displacement of VASP by these drugs (E. Vignal, K. Rottner and J. V. Small, unpublished observations). Alternative approaches are hence required to test whether a direct interaction of Ena/VASP proteins with the barbed ends of lamellipodial actin filaments is indeed relevant for their recruitment to these sites.

The targeting of the EVH1 domain of Ena/VASP to lamellipodia tips is likely due to its association with proteins harbouring proline-rich regions that localise at this site. Among them are the Abl-interacting proteins (Abis), which have been implicated in cytoskeletal rearrangement following the stimulation with growth factors (78-80). Similar to the behaviour of Ena/VASP proteins during lamellipodia dynamics, Abis exclusively localises at the tips of spreading lamellipodia and filopodia (81). Moreover, Abi2 directly interacts with Mena both *in vitro* and *in vivo* and co-localises with it at the leading edge (82). Taken together, these findings suggest that Abi proteins may be responsible for the EVH1-mediated targeting of Ena/VASP proteins to the lamellipodial tips. Recently, it has been shown that proteins of the Scar/WAVE family localise to the edge of lamellipodia in mouse fibroblasts and human melanoma cells (83, 84). Since WASP (an homologue of Scar/WAVE proteins) interacts with Ena/VASP proteins via its proline-rich region (19) and Scar/WAVE proteins also contain proline-rich sequences in their central region, they may be potential candidates for recruiting Ena/VASP proteins at the leading edge. Formal demonstration that Abis and Scar/WAVE are necessary for

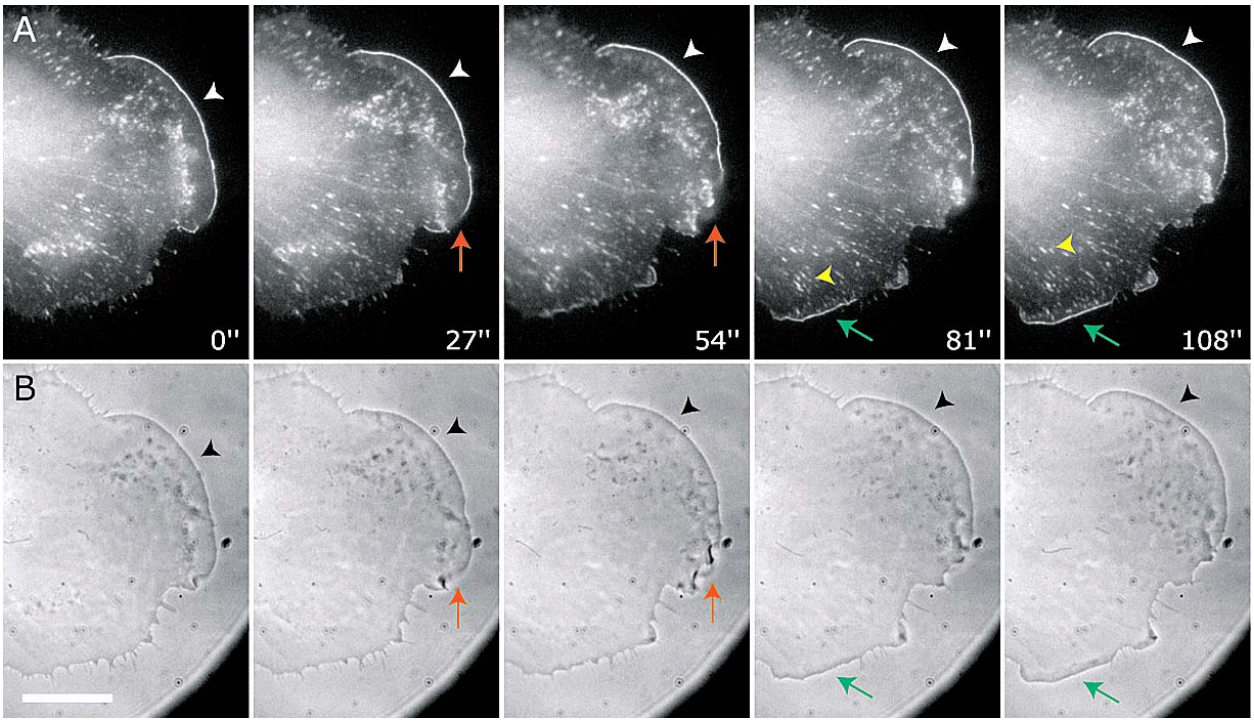

Figure 4. Dynamics of GFP-VASP during lamellipodia protrusion in B16F1 cells. Panels in (A) show GFP-VASP, whereas panels in (B) show the corresponding phase contrast images. GFP-VASP localises at the tip of a spreading lamellipodium (arrowheads), where it localises at the onset of lamellipodial spreading (green arrows). In contrast, GFP-VASP disappears from such location at the onset of the retraction of the lamellipodium (red arrows). GFP-VASP also localises at the small focal adhesions formed by B16F1 cells upon interaction with the substrate (yellow arrowheads). Scale bar represents 10 µm.

the targeting of Ena/VASP proteins to lamellipodial tips should be obtained, for example, through the determination of Ena/VASP protein localisation in Abis- and Scar/WAVE-deficient cells. Finally, the crystal structure of the EVH1 domain revealed that it strongly resembles that of pleckstrin homology (PH) domains (85, 86). Since PH domains are responsible for the interaction of proteins with plasma membrane lipids PIP2 and PIP3 (see 87), it is conceivable that the targeting of Ena/VASP proteins to the leading edge may be mediated by their association with phosphoinositides.

The implication of Ena/VASP proteins in actin filament dynamics at the leading edge was initially shown by the finding that the velocity of lamellipodia protrusion is proportional to the levels of GFP-VASP at the tips of these structures (74). Moreover, the distribution and dynamics of GFP-profilin at the tips of lamellipodia accurately reflects that of Ena/VASP proteins (61), whereas the injection of cross-linked profilin:actin complexes into fibroblast blocks lamellipodia spreading (88). Consistent with the role of Ena/VASP proteins and profilin:actin in supporting actin filament elongation at *Listeria* surface (see above), these studies clearly demonstrate that Ena/VASP proteins are also essential for supporting efficient actin filament elongation at the leading edge. Accordingly, the displacement of Ena/VASP from lamellipodia reduces the speed of lamellipodia advancement in fibroblasts, whereas the constitutive targeting of these proteins to the plasma membrane significantly increases the speed of this process (58). In this context, it should be noted that in another cellular system, namely mouse cardiac fibroblasts, the deletion of VASP leads to enhanced cell spreading, formation of highly motile lamellipodia and sustained activation of the small GTPase Rac (89). Although a detailed analysis of lamellipodia speed was not provided in this study, it implies that Ena/VASP proteins may down-regulate the formation of lamellipodia perhaps by affecting the Rac signalling pathway. Moreover, although Ena/VASP proteins are functionally interchangeable in the *Listeria* system (35, 55) the presence of physiological levels of Mena and EVL in VASP$^{-/-}$ mouse cardiac fibroblasts (89) argue for different functions of Ena/VASP proteins in various cellular contexts.

Other than supporting actin filament elongation at lamellipodial tips, Ena/VASP proteins appear to regulate the protrusion of lamellipodia by changing the architecture of the lamellipodial network of actin filaments. In particular, in the absence of these proteins actin filaments at the leading edge are shorter and highly branched than those in control cells, whereas the artificial enrichment of Ena/VASP proteins at the plasma membrane causes the formation of long and less branched actin filaments which mostly run perpendicular to the direction of lamellipodia advancement (58). Since the velocity of protrusion of lamellipodia lacking Ena/VASP proteins is much smaller than that of lamellipodia rich in Ena/VASP proteins, it was proposed that Ena/VASP proteins regulate the speed of lamellipodia protrusion by changing the length and

orientation of actin filaments abutting the plasma membrane (58). Given that maximal lamellipodial advancement is achieved when the growing actin filaments abutting the inner side of the plasma membrane do so at an optimal angle and having an optimal length (see 90), it is not clear how actin filaments running mostly parallel to the plasma membrane (which, according to the theory of Molginer and Oster, should not be able to generate any productive force to support lamellipodial advancement) can support the fast lamellipodial speed observed when Ena/VASP proteins are constitutively targeted to the plasma membrane. Thus, while the effect of Ena/VASP proteins on the lamellipodial actin network is consistent with the ability of these proteins to counteract the function of capping proteins and regulate the Arp2/3 complex-dependent actin filament branching (54, 57, 58), the lack of information concerning the dynamics of lamellipodia prior to fixation makes it difficult to establish a clear correlation between speed of lamellipodia protrusion and actin filament architecture.

5.3.2. Ena/VASP protein localisation and function in filopodia and focal adhesions

During the motility of melanoma and fibroblastic cell lines, Ena/VASP proteins localise at the tips of filopodia, thin bundles of actin filaments that form within a spreading lamellipodium and project beyond its edge (74). Ena/VASP proteins also localise at the tips of filopodia-like structures that form as the prelude to the assembly of cadherin-based intercellular junctions and are also essential for this process (91). Little is known about the mechanisms underlying the targeting of Ena/VASP proteins to the tips of filopodia. However, since Abis and Scar/WAVE proteins also localise to these structures (81, 83), these proteins may contribute to the EVH1 domain-dependent targeting of Ena/VASP proteins to filopodia tips. It cannot be excluded that other yet unidentified proteins containing EVH1-binding motifs or the association of Ena/VASP proteins with actin filaments or with phospholipids contribute to their localisation at these subcellular sites. Concerning the function of Ena/VASP proteins in filopodia dynamics, the localisation of profilin at the tips of these structures (61) suggests that Ena/VASP proteins may support the elongation of actin filaments in filopodia by recruiting profilin-actin complexes. Moreover, in light of the counteracting function of Ena/VASP proteins on capping protein, it has recently been proposed that these proteins may antagonise capping protein during filopodia formation thus allowing the sustained actin filament elongation that is required for the elongation of filopodia (92). Whether Ena/VASP proteins can also influence the architecture of actin filaments within filopodia remains to be determined. Importantly, the function of Ena/VASP proteins in filopodia formation appears to be linked to its association with the protein IRSp53 (28), although a recent study had shown that both the localisation of IRSp53 as well as filopodia formation can occur independently of Ena/VASP proteins (93).

Focal adhesions are highly complex regions of the ventral side of a cell that serve to anchor it to the substratum via integrin-based interactions (see 94). In contrast to lamellipodia and filopodia, the mechanism underlying the targeting of Ena/VASP proteins to focal adhesions (figure 5) exclusively depends on EVH1-mediated interactions since the injection of a peptide spanning one proline-rich repeat of ActA (44) and the deletion of the EVH1 domain, but not of the EVH2 domain or the F-actin-binding site within it, displace Ena/VASP proteins from these sites (75, 76). Among the 50 or more components that have been associated with focal adhesions, only vinculin and zyxin have been demonstrated to bind to Ena/VASP proteins both *in vitro* and *in vivo* (13, 17). Consistent with these findings, the analysis of the dynamics of both vinculin and zyxin during spontaneous or induced disassembly of focal adhesions indicates that both proteins contribute to the targeting of Ena/VASP proteins to these sites (95). More recently, a zyxin-like protein, TES, has been shown to localise at focal adhesions and to associate with VASP and Mena (96), suggesting that it may also be implicated in the localisation of Ena/VASP proteins at focal adhesions. As far as the assembly of focal adhesions is concerned, Ena/VASP proteins appear not to play an essential role in this process since the lack of these proteins does not cause gross alterations in the composition and morphology of the focal adhesions (75). Given that the dynamics of focal adhesions was not analysed in Ena/VASP-deficient cells, we cannot exclude that these proteins regulate one or more aspects of this process. The findings that the adhesion of platelets to fibrinogen is enhanced in the absence of VASP and that this protein regulates the inactivation of integrin support this possibility (97-99).

6. FUNCTIONS OF ENA/VASP PROTEINS IN COMPLEX CELLULAR SYSTEMS

While studies on *Listeria* motility, lamellipodia dynamics, T cell activation and phagocytosis have clearly demonstrated that Ena/VASP proteins enhance actin filament dynamics, other studies suggest that Ena/VASP proteins may negatively influence actin-based processes. In *Drosophila* and mice, the deletion of Ena/VASP proteins leads to defects in the migration of axonal growth cones, which fail to stop at precise choice points but migrate farther (100, 101). Ena is also required for the repulsion of growth cones mediated by the Roundabout receptor (102), whereas $VASP^{-/-}$ platelets tend to aggregate faster than their wild-type counterparts (97, 98). Finally, it has been shown that fibroblasts lacking both Mena and VASP (but expressing sub-optimal levels of EVL) move faster in random motility assays (75). Although it is clear that Ena/VASP proteins act as negative regulators in all these processes, it must be pointed out that cellular processes such as cell motility and platelet aggregation do not exclusively depend on actin filament assembly but are the result of the integration of many sub-cellular events including activation of the contractile apparatus and the formation of cell-matrix interactions. Therefore, it is difficult to establish a direct correlation between function of Ena/VASP proteins and cell motility or platelet aggregation. On the other hand, when only actin filament assembly is at play such as during lamellipodial protrusion and *Listeria* motility, Ena/VASP proteins unequivocally function as promoters of actin filament elongation. To understand how this negative influence is generated, future

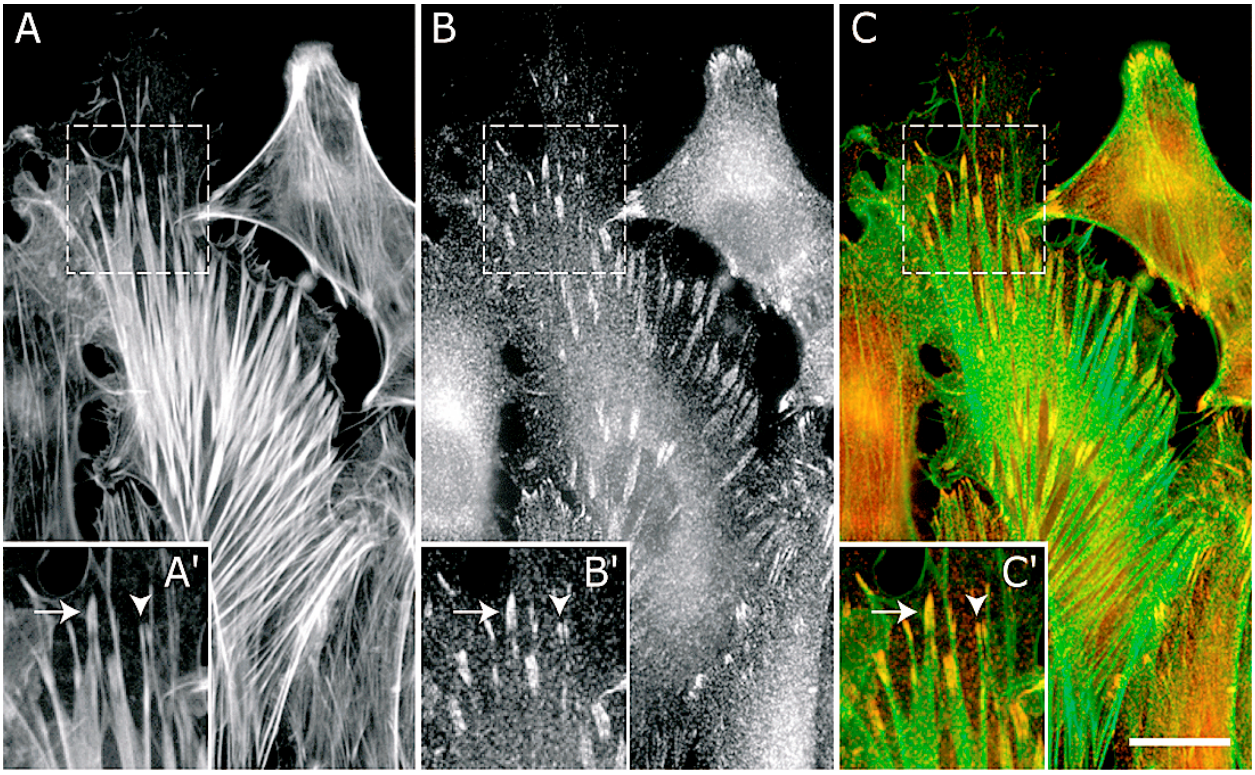

Figure 5. Immunolabelling of Ena/VASP proteins at focal contacts in cultured cells. Rat embryo fibroblasts REF-52 were grown on glass cover slips, fixed and labelled with fluorescent phalloidin (A-A', pseudo-coloured in green in C-C') and with the monoclonal antibody 49C2B12 against Mena (B-B', pseudo-coloured in red in C-C'). Mena robustly localises to focal adhesion sites (arrow and arrowhead in B') which correspond to the terminal ends of prominent stress fibres (arrow and arrowhead in A') which across almost the entire length of the cell. Scale bar represents 20 μm (for A-C) and 30 μm (for A'-C').

studies should be aimed at, for instance, determining whether the lack of Ena/VASP proteins affects the regulation of the contractile apparatus, the dynamics of focal adhesions and the generation and maintenance of cell polarity.

7. REGULATION OF ENA/VASP PROTEIN FUNCTION

Ena/VASP proteins are targets for Ser/Thr and tyrosine protein kinases suggesting that their function(s) and sub-cellular localisation are regulated by phosphorylation. As discussed above, the phosphorylation of VASP influences its interaction with actin filaments and monomers and also affects the ability of VASP to promote actin filament polymerisation (32, 35, 36). In addition, PKA-mediated phosphorylation of EVL and VASP abolishes their interaction with SH3 domains of c-Abl and nSrc *in vitro* (11, 103). Similarly, the phosphorylation of the tyrosine residues within Ena reduces the interaction of this protein with c-Abl and Src (34). By contrast, the association of Ena/VASP proteins with SH3 domain of Lyn, the WW domain of FE65 and with the cytoskeletal proteins zyxin, profilin and vinculin is insensitive to the phosphorylation state of Ena/VASP proteins (11, 36).

At the cellular level, Ser/Thr phosphorylation appears not to be required for the targeting of Ena/VASP proteins to focal adhesions and tips of lamellipodia, since a Mena mutant that cannot be phosphorylated properly localises to these sites (76). Conversely, tyrosine phosphorylation is required for the function of Ena since a phosphorylation-deficient Ena protein is unable to fully restore viability of *Drosophila* Ena mutants (34). In addition, the inhibition of platelet and integrin activation appears to correlate with the PKA-induced phosphorylation of VASP (104-106). In other cellular systems, the detachment of cells from their substrata causes an increase in the activity of PKA and the corresponding phosphorylation of VASP and Mena (103). Moreover, VASP and Mena are de-phosphorylated following re-attachment and acquire a phosphorylation state during cell spreading (103, 107). The PKG-dependent phosphorylation of VASP leads to the depletion of these proteins from focal adhesions, the disruption of stress fibres and inhibits cell migration in a Boyden chamber assay (108). In the *Listeria* system, Ena/VASP mutants that constitutively mimic the fully phosphorylated form of these proteins significantly enhance bacterial motility (55). Since *Listeria* is tighly linked to its actin tail (65) probably via Ena/VASP-F-actin interactions (66), phosphorylation of Ena/VASP proteins may weaken their interaction with actin filaments thus leading to faster bacterial movement.

Recent biochemical studies showed that Abl-induced tyrosine phosphorylation of Mena can be promoted by

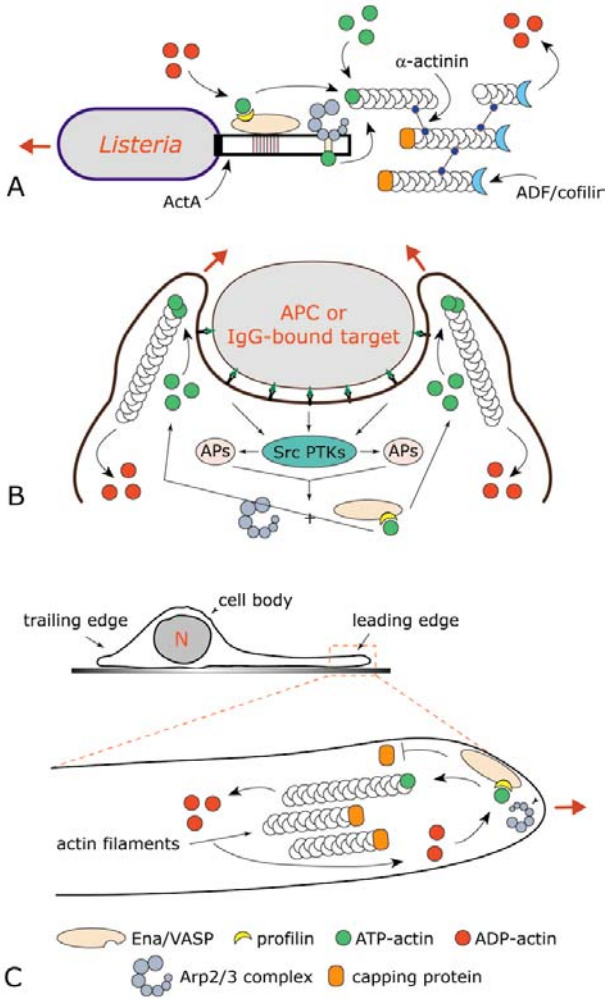

Figure 6. Modes of action of Ena/VASP proteins during actin based processes. (A) During *Listeria* motility Ena/VASP proteins are targeted to the bacterial surface via the interaction with the proline-rich region of ActA. At this site, Ena/VASP proteins recruit profilin:actin complexes that are then delivered to the growing actin filament barbed ends thus supporting efficient bacterial motility (red arrow). (B) The interaction between an antigen-presenting cell (APC) or a IgG-opsonised particle with T cells and macrophages, respectively, triggers a cascade of events leading to the activation of Src protein kinases (PTKs), the recruitment of several adapter proteins (APs), the activation of the Arp2/3 complex and the targeting of Ena/VASP proteins to upstream components of this signalling pathways. In this context, Ena/VASP proteins act by targeting profilin:actin to the fast growing actin filament ends that support the forward movement (red arrows) of the plasma membrane surrounding the APC or the IgG-bound target. (C) During cell motility, Ena/VASP proteins are recruited to the tips of the protruding lamellipodium where, in analogy to their function in the previous two actin-based processes, they recruit profilin:actin complexes to support the fast actin filament elongation necessary to sustain the rapid advancement of the lamellipodium (red arrow). Moreover, Ena/VASP proteins may also favour actin filament growth by blocking the interaction between capping proteins and the filaments most proximal to the plasma membrane. Please note that to highlight the function of Ena/VASP proteins in these processes and simplify the cartoons, the Arp2/3 complex-induced actin filament nucleation and branching as well as the effect of Ena/VASP proteins on the branching activity of this complex are not depicted.

Abi (82). Abi may function as a linker between Abl and Mena by simultaneous binding to both proteins via its SH3 domain (it interacts with the PRR of Abl) and PRR (it binds to the EVH1 domain of Mena) and may also stimulate the kinase activity of Abl (109) thus providing a potential regulatory mechanism of Ena/VASP protein phosphorylation. It should be pointed out that Ena and Abl are also targets for the receptor protein tyrosine phosphatase LAR (Leukocyte Antigen Related) (101, 110). Since the catalytic activity of Abl is stimulated by tyrosine phosphorylation (see 111), the interplay between Abl and LAR may be required for the regulation of the tyrosine phosphorylation of Ena/VASP proteins. Finally, since the interaction between VASP and vinculin is augmented by PIP2 *in vitro* (112), the function of Ena/VASP proteins may also be regulated by this phosphoinositide.

8. PERSPECTIVES

It is now well established that Ena/VASP proteins regulate actin cytoskeleton dynamics by controlling the magnitude and persistence of actin filament elongation (figure 6). In addition, these proteins can also counteract the activity

of capping proteins and regulate the Arp2/3-driven branching of actin filaments, thus influencing the final architecture of the actin cytoskeleton. In spite of this wealth of knowledge, many questions remain to be answered. For instance, how are Ena/VASP proteins targeted to the tips of lamellipodia and filopodia? Do phosphoinositides play a role in this process and in the function of Ena/VASP proteins? How does Ser/Thr and Tyr phosphorylation regulate the functions of this protein family?

Since the absence of Ena/VASP proteins in different fibroblastic cell lines leads to different effects on lamellipodial dynamics and cell motility (58, 89), it should be established to which extent Ena/VASP proteins are functionally interchangeable and what specific functions can be ascribed to single members of this protein family. These questions can be addressed, for instance, by the specific suppression of one or more of Ena/VASP family members in different cellular contexts via small interfering RNAs (see 113). Moreover, in contrast to previous reports, it has been shown that the VASP- and Mena-deficient cell line MV^{D7} expresses indeed detectable levels of EVL (64). Although the presence of EVL has no obvious effects on *Listeria* motility (there is no difference between the speed of wild-type bacteria and that of a *Listeria* mutant which is unable to recruit Ena/VASP proteins, 55), it will be important to determine whether, and to which extent, the presence of EVL can affect actin cytoskeleton dynamics in general. Finally, cell type- and tissue-specific immuno-precipitations of Ena/VASP proteins in combination with advanced techniques for protein analysis should provide a more complete overview of the binding partners of these proteins.

Beyond their role in actin cytoskeleton dynamics, do Ena/VASP proteins regulate other cellular processes? In this context, it is interesting to point out that Ena/VASP proteins have been implicated in the activation of the serum response factor (SRF) and the subsequent activation of gene expression (114, 115). Interestingly, SRF controls the expression of genes encoding for some cytoskeletal components such as β-actin and vinculin (114), raising the possibility that Ena/VASP proteins influence the physiology of the actin cytoskeleton via the regulation of the expression of some cytoskeletal-specific genes. Finally, either the overexpression or the deficiency of VASP appears to cause neoplastic transformation (116), suggesting that Ena/VASP proteins may play a role in cancer genesis and/or progression. The answers to these questions will provide new insights into both the regulation Ena/VASP protein functions and of actin cytoskeleton remodelling in general.

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