Ena/VASP Proteins Enhance Actin Polymerization in the Presence of Barbed End Capping Proteins*S

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Ena/VASP proteins influence the organization of actin filament networks within lamellipodia and filopodia of migrating cells and in actin comet tails. The molecular mechanisms by which Ena/VASP proteins control actin dynamics are unknown. We investigated how Ena/VASP proteins regulate actin polymerization at actin filament barbed ends in vitro in the presence and absence of barbed end capping proteins. Recombinant His-tagged VASP increased the rate of actin polymerization in the presence of the barbed end cappers, heterodimeric capping protein (CP), CapG, and gelsolin-actin complex. Profilin enhanced the ability of VASP to protect barbed ends from capping by CP, and this required interactions of profilin with G-actin and VASP. The VASP EVH2 domain was sufficient to protect barbed ends from capping, and the F-actin and G-actin binding motifs within EVH2 were required. Phosphorylation by protein kinase A at sites within the VASP EVH2 domain regulated anti-capping and F-actin bundling by VASP. We propose that Ena/VASP proteins associate at or near actin filament barbed ends, promote actin assembly, and restrict the access of barbed end capping proteins.

The vertebrate Ena/VASP proteins Mena, VASP, and Evl play important functions in regulating the cytoskeleton during cell motility, axon outgrowth, and guidance (1, 2) and for actin-based motility of the pathogenic bacterium Listeria monocytogenes (3–6). Ena/VASP proteins influence the dynamics of lamellipodia and formation of filopodial protrusions in fibroblasts and neuronal growth cones (7–9). In Dictyostelium, VASP is required for efficient chemotaxis and formation of filopodia (10). Ena/VASP proteins regulate the architecture of actin filaments in the actin tails of motile Listeria (11) or those associated with beads coated with the Listeria ActA protein (12, 13). The mechanisms by which Ena/VASP proteins regulate these diverse actin-dependent events are unknown.

Clues to a possible mechanism for Ena/VASP proteins during cell migration came from electron microscopic studies of actin filaments in lamellipodia of fibroblasts and neuronal growth cones. Depletion of Ena/VASP proteins from their normal locations in fibroblasts or neurons promoted formation of dense actin networks with short, highly branched filaments. In contrast, enrichment of Ena/VASP proteins at the plasma membrane resulted in sparse networks containing primarily long, unbranched filaments, which in growth cones coalesced into filopodia (8, 9). These studies support the hypothesis that Ena/VASP proteins influence actin networks by promoting formation of long, unbranched actin filaments in the cell periphery.

Ena/VASP proteins could influence the length of actin filaments and the extent of filament branching at the cell periphery via several mechanisms: by increasing the rate of actin filament elongation, reducing the frequency of forming branched actin filaments, increasing the dissociation rate of branched filament junctions, or decreasing barbed end capping activity. Biochemical experiments in which recombinant VASP enhanced actin polymerization in the presence of heterodimeric capping protein (CP) supported the hypothesis that Ena/VASP proteins promote the formation of long, unbranched actin filaments, in part, by protecting actin filament barbed ends from capping (9).

To determine the mechanism by which Ena/VASP proteins regulate actin polymerization and barbed end capping, we studied His6-tagged VASP and several mutant forms of VASP in biochemical assays of actin assembly in the presence of bacterially expressed capping proteins and profilin. Structural features of the VASP EVH2 domain essential for regulating actin assembly in the presence of barbed end capping proteins in vitro were similar to those required for Mena to restore normal motility to Ena/VASP-deficient fibroblasts (14).

EXPERIMENTAL PROCEDURES

Recombinant Protein Expression and Purification—All VASP proteins were recombinant, His6-tagged and purified from Escherichia coli. Plasmids to express N-terminal His6-tagged murine VASP and mutant VASP proteins were constructed from PCR fragments cloned into pQE-80L (Qiagen). The wild-type and mutant VASP proteins used in this study are shown in schematic form in supplementary materials Fig. 1. VASP proteins were expressed in E. coli strain BL21 (DE3) CodonPlus and purified by chromatography on TALON resin (BD Biosciences) and Superdex-200 in MKEI-200 buffer (20 mM imidazole, pH 7.0, 200 mM KCl, 1 mM EGTA, 2 mM MgCl2, and 1 mM DTT). VASP proteins were stored on ice and used within 2 weeks of purification. Recombinant murine CP (αβ2) was purified as described (15). Plasmids for expression of human profilin 1, profilin 1-Y6D, and profilin 1-R88E were constructed by D. Kaiser and J. Lu and were gifts from the laboratory S1–S3.

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of T. D. Pollard; profilins were purified as described (16). The actin
binding and polyproline binding activities of wild-type and mutant
profilins were verified. Recombinant gelsolin and CapG were gifts of M.
Weeds and T. Pollard, respectively. Gelsolin-actin complex was pre-
pared as a 1:2 molar ratio of gelsolin and actin in 20 mM imidazole, pH
7.0, 100 mM KCl, 1 mM EGTA, 2 mM MgCl2, 1 mM DTT, and 0.2 mM
CaCl2. Actin was prepared from rabbit muscle (17) and gel filtered on
Sephacryl S200 equilibrated in 2 mM Tris/HCl, pH 8.0, 0.2 mM ATP, 0.1
mM DTT, and 0.2 mM CaCl2. Pyrenyl-actin was prepared as described
(18). All proteins except actin were quantified from absorbance at 280
nm using a Shimadzu UV-1601PC (Shimadzu Scientific Instruments, Inc).
Fluorescence was excited at 365 nm; the emission was monitored at
386 nm using extinction coefficients predicted from the amino acid sequence
(Protein, DNASTar software): VASP, 32,650 M-1 cm-1; considered to be a tetramer
(based on analytical ultracentrifugation experiments below); CP, 76,290 M-1 cm-1;
and profilin, 18,100 M-1 cm-1. Actin was quantified from absorbance at 290 nm
and the extinction coefficient of 26,600 M-1 cm-1.

Other Proteins—Spectrin F-actin seeds (SAS) were prepared from human erythrocytes as described (19). The concentration of SAS
determined from the initial rate of actin polymerization using
human erythrocytes as described (19). The concentration of SAS was
Seeded polymerization reactions contained 0.5–2 µM actin (5% pyrene-labeled), 0.2 mM SAS, 4 mM CP, VASP/mutant VASP protein, and profilin/mutant profilin, as indicated, in 20 mM imidazole, pH 7.0, 100 mM KCl, 2 mM MgCl2, 1 mM EGTA, 0.2 mM ATP, and 0.1 mM DTT (MKEI-100 buffer). SAS were omitted from reactions monitoring spontaneous actin polymerization. Fluorescence of
pyrenyl-actin (excitation at 365 nm, emission at 386 nm) was monitored for
600 s at 25 °C. All components except actin and SAS were mixed in
MKEI-100 buffer; reactions were initiated by the simultaneous addition
of actin (primed with 1 mM EGTA and 0.1 mM MgCl2 for 90 s) and SAS.
The delay between mixing reactants and recording fluorescence was
<16 s. In experiments with profilin, VASP and profilin were incubated in
MKEI-100 buffer for 5 min prior to adding other components. 
Fluorescence was converted to the molar concentration of F-actin from
the fluorescence of completely polymerized and unpolymerized actin,
assuming a critical concentration of 0.1 M. Initial rates of actin assembly
were determined from linear fits of data collected during the initial
60 s. In control experiments, VASP did not alter the fluorescence of
either pyrenyl-G-actin or pyrenyl-F-actin.

Effect of VASP on Spontaneous Polymerization of G-actin—The effect of VASP on the concentration of G-actin present in steady state solutions of 1
µM actin in MKEI-100 buffer was determined in reactions containing varying amounts of VASP with or without 10 mM CP in MKEI-100 buffer. 
Samples were incubated for 16 h at room temperature to reach equilibri-
um. The reactions were overlaid on a 10% sucrose cushion and sub-
jected to centrifugation for 20 min at 80,000 × g in a TLA 120.1 rotor at
20 °C. Aliquots of the supernatant fraction were subjected to SDS-
PAGE and stained with Coomassie Brilliant Blue. The amount of G-actin present in the supernatant fraction was quantified from the 
Coomassie-stained gels using ImageJava software and gels containing
known amounts of actin.

Quantitation of Actin Filament Barbed Ends—To determine if
barbed ends were created de novo, reactions containing VASP or EVH2 and
either 1 µM actin (for VASP) or 0.5 µM actin (for EVH2) were prepared without pyrenyl-actin as described for seeded polymerization assays.
Profilin (10 µM) was included as indicated. After 90 s, reactions were
diluted 25-fold into 0.5 µM actin (5% pyrene-labeled), and fluores-
cence was monitored for 600 s.

Actin Depolymerization Assays—F-actin (1 µM, 50% pyrene-labeled)
was incubated with 17 nm CP and varying amounts of VASP for 5 min. 
Care was taken to minimize filament breakage during mixing by using
wide-bore pipette tips, triturating a defined number of times, and transferring F-actin solutions slowly. Aliquots were diluted 20-fold into
stirring MKEI-100, and fluorescence of pyrenyl-actin was monitored for
600 s.

Phosphorylation of VASP by Protein Kinase A (PKA)—VASP at a
final concentration of 2 µM was incubated for 30 min at 30 °C with 200
units/ml of cAMP-dependent protein kinase, catalytic subunit (New
England Biolabs) in 50 mM Tris/HCl, pH 7.5, 10 mM MgCl2, and 1 mM
ATP. Mock-treated VASP was prepared similarly in a reaction without
added PKA.

Analytical Ultracentrifugation Analyses—For velocity analytical ultracentrifugation, 4 µM VASP in 150 mM NaCl, 0.5 mM EGTA, 10 mM
NaH2PO4, pH 7.0, 0.5 mM DTT, was centrifuged at 40,000 × g at 20 °C
in a Beckman Protomelab XL-A rotor; 280 nm absorbance was moni-
tored every minute by continuous scan at 0.003 cm. Protein partial
specific volume, buffer density, and buffer viscosity were determined
using Sednterp. Scans 1–200 were analyzed using Sedfit87 (www.
analyticalultracentrifugation.com). For sedimentation equilibrium ultracentrifugation, three concentrations of VASP (4, 2, and 1 µM) were
centrifuged at 7,000, 10,000, and 14,000 × g for 15, 10, and 10 h,
respectively, in an AN-60 rotor. Scans at 280 nm and 0.001-cm steps were recorded every hour. Winnmac, Winrediet, and Winnonlin software were used to analyze and fit the data, assuming a single species. The resulting fit gave a of 7.99 and root mean square deviation of 0.00409 with no systematic deviation of residuals.

Microscopy of Actin Filaments—Actin filaments were stained with
rhodamine-phalloidin (7,000, 10,000, and 14,000 × g for 15, 10, and 10 h,
respectively, in an AN-60 rotor. Scans at 280 nm and 0.001-cm steps were recorded every hour. Winnmac, Winrediet, and Winnonlin software were used to analyze and fit the data, assuming a single species. The resulting fit gave a of 7.99 and root mean square deviation of 0.00409 with no systematic deviation of residuals.

RESULTS

VASP Protects Actin Filament Barbed Ends from Capping—To understand how Ena/VASP proteins regulate actin filament assembly in the presence of barbed end-capping proteins, we studied actin polymerization from SAS in the presence of recombinant CP and His8-tagged VASP. We confirmed our previous findings that VASP increased the rate of actin polymerization from SAS in the presence of CP in a dose-dependent manner (9) (Fig. 1, A and B). The ability of VASP to enhance actin polymerization in the presence of CP, defined here as anti-capping activity, also depended on the concentration of capping protein (Fig. 1C). The apparent inhibition of capping by VASP could occur by several mechanisms: 1) VASP could compete directly with CP for binding barbed ends; 2) VASP could promote formation of new filament barbed ends via de novo filament nucleation or severing; or 3) VASP could increase the rate of filament elongation. A fourth possibility that VASP binds CP and prevents its interaction with barbed ends was ruled out in previous studies (9).

To determine if VASP anti-capping activity involved enhanced filament elongation, we examined the effects of VASP on filament elongation in actin polymerization assays seeded by SAS. VASP increased slightly the initial rate of actin polymerization from SAS (Fig. 1D), suggesting that VASP enhances the rate of actin subunit association or decreases the rate of subunit dissociation. The increased rate of actin polymerization was apparent after a short lag period (~30 s) indicated by the slight upward curvature in the reaction time courses. This lag period may reflect the fact that short actin filaments nucleated by SAS are required for VASP to associate with elongating filaments.

The ability of VASP to increase the rate of actin polymerization did not result from de novo formation of actin filaments. Under the experimental conditions, spontaneous actin nucleation activity of VASP was negligible at concentrations below 150 nM (Fig. 1E). An increased rate of spontaneous actin polymerization was apparent at higher concentrations of VASP (>200 nM) (Fig. 1E), however, this activity required a prolonged lag period and did not account for the increased initial rates of actin polymerization in reactions containing VASP.
that VASP did not generate new actin filaments within the time required to determine initial rate measurements, aliquots of polymerization reactions containing SAS and varying amounts of VASP were removed at 90 s and diluted into 0.5 μM actin to observe filament elongation at barbed ends selectively. The rate of actin polymerization after dilution was independent

FIG. 1. VASP inhibits barbed end capping by CP. A, VASP increases the rate of actin polymerization in the presence of CP in a dose-dependent manner. Plotted are actin polymer concentrations versus time in reactions containing 1 μM actin, 4 nM CP, 0.2 nM SAS, and VASP as indicated beside each curve: no VASP, red; 12 nM, orange; 25 nM, green; 50 nM, blue; 100 nM, pink; 150 nM, dark blue; 200 nM, violet. The curve labeled SAS alone (black) contained neither VASP nor CP. B, dependence of the initial rate of actin polymerization in the presence of 4 nM CP on the concentration of VASP. Initial rates were calculated from a linear fit to the first 60 s of each time course in panel A and normalized to the initial rate in reactions containing SAS alone. C, anti-capping activity is dependent on the concentrations of both CP and VASP. Reactions contained 1 μM actin, and varying amounts of CP and VASP as indicated: no VASP, filled circles; 16 nM, open circles; 50 nM, filled squares; 82 nM, open diamonds. Initial rates were calculated from a linear fit to the first 15–60 s (depending on the [CP]) of each time course and normalized to that obtained in reactions containing no CP. D, VASP increased the rate of actin polymerization from SAS. Plotted are actin polymer concentrations versus time in reactions containing 1 μM actin, 0.2 nM SAS, and VASP as indicated: no VASP, black; 25 nM, orange; 100 nM, green. E, spontaneous actin polymerization by VASP is negligible under the conditions used for anti-capping assays. Plotted is the actin polymer concentration versus time in reactions containing 1 μM actin and VASP as indicated: 50 nM, blue; 100 nM, pink; 150 nM, dark blue; 200 nM, violet. F, VASP does not promote de novo formation of new actin filament-barbed ends. Reactions containing 1 μM actin, 0.2 nM SAS, and VASP (50 nM VASP, green; 100 nM VASP, blue; 200 nM, violet) and with 10 μM profilin (25 nM VASP, orange; 50 nM VASP, red) as indicated were incubated for 90 s at room temperature followed by 25-fold dilution in 0.5 μM actin (5% pyrenyl-actin). Plotted is the actin polymer concentration versus time after dilution.
of VASP (Fig. 1F), indicating that filament-barbed ends were not generated. These results confirm previous findings that VASP exhibits negligible actin nucleation activity at physiological salt conditions (3, 9, 25–27) and rules out that the apparent competition between capping proteins and VASP in seeded polymerization assays results from spontaneous actin nucleation as suggested by Samarin and colleagues (13). The mammalian Ena/VASP proteins, Mena and Evl, also exhibited anti-capping activity and enhanced the rate of actin filament elongation under conditions where actin nucleating activity was negligible (data not shown).

VASP anti-capping activity depended on the concentrations of VASP and CP (Fig. 1, B and C), suggesting that CP and VASP compete for binding barbed ends. VASP also protected barbed ends from two other proteins with barbed end capping activity: gelsolin-actin complex and CapG (Fig. 2, A and B). The ability of VASP to inhibit capping by several different barbed end-binding proteins suggests that VASP prevents capping by directly associating with barbed ends. In support of this hypothesis, VASP decreased the amount of G-actin present in actin solutions containing CP under steady state conditions, indicating that VASP blocked CP from binding barbed ends without interfering with actin subunit addition (Fig. 2C). VASP alone increased slightly the amount of G-actin at steady state (Fig. 2C), consistent with a low-affinity interaction of VASP and G-actin (28).

VASP also competed with CP for binding pre-formed actin filaments. Actin filaments capped by CP depolymerized slowly when diluted below the barbed end critical concentration, as expected. VASP competed with CP, resulting in increased rates of depolymerization of uncapped actin filaments, indicating that VASP does not sever filaments (data not shown).

The EVH2 Domain of VASP Is Sufficient to Protect Barbed Ends from CP—The C-terminal EVH2 domain of VASP, which contains separate motifs that interact with G-actin (28), F-actin (25, 29), and confer VASP tetramerization (29), was more active than full-length VASP in enhancing actin polymerization in the presence of CP (Fig. 3, A and B). Actin nucleation activity by EVH2 was slightly higher than that of VASP (Fig. 3C), however, anti-capping activities of EVH2 and VASP could be compared in assays using 0.5 μM actin, where actin nucleation activity by both proteins was negligible. We confirmed
that EVH2 did not promote formation of new filaments under these conditions using dilution assays that selectively monitor elongation at actin filament barbed ends (Fig. 3D). Thus, the VASP EVH2 domain contains the structural elements sufficient for anti-capping activity. The EVH2 domain of Mena similarly exhibited potent anti-capping activity (data not shown).

**Interactions via G-actin and F-actin Binding Motifs of VASP Are Required for Anti-capping Activity**—The F-actin and G-actin binding motifs are required for Ena/VASP function during whole cell motility (14). To determine if VASP anti-capping activity depended on interactions via its G-actin and F-actin binding motifs, we examined the anti-capping activity of mutant proteins that either lack or alter these motifs. Mutant VASP with a deletion at the F-actin binding motif (VASPΔFAB, Δ256–273) did not protect barbed ends from CP, even when tested at concentrations exceeding 400 nM (supplementary materials Fig. 2A). Similarly, VASP with mutations that disrupt the G-actin binding site (VASP-GAB; R232E,K233E) (28) did not protect barbed ends from CP when used at concentrations up to 400 nM (supplementary materials Fig. 2B).

To determine if VASP binding to free G-actin was required for anti-capping activity, we examined the dependence of anti-capping activity on the concentration of G-actin. VASP anti-capping activity was independent of the concentration of G-actin (supplementary materials Fig. 2C), indicating that anti-capping activity likely does not involve interactions of VASP and free G-actin. Instead, we suggest that VASP anti-capping activity requires interactions of the G-actin binding motif with actin subunits incorporated in filaments, possibly the terminal actin subunits situated at barbed ends.
VASP Tetramers Are Required for Anti-capping Activity—VASP is predicted to assemble as a tetramer (29–31). We confirmed that full-length VASP is a tetramer using equilibrium sedimentation ultracentrifugation. A molecular mass of 173.2 kDa was calculated from the experimentally determined effective reduced molecular weight and partial specific volume of 0.7219 ml/g. The predicted molecular mass for His6-tagged monomeric VASP is 41.2 kDa, corresponding to a 164.8-kDa tetramer. No systematic deviation between experimental data and curve fit was present, suggesting that VASP is a stable tetramer. Sedimentation velocity ultracentrifugation experiments for VASP showed a single peak at 4.7 S with a frictional coefficient ($f / f_0$) of 2.02, consistent with VASP being an asymmetric, elongated molecule (supplementary materials Fig. 3A).

To determine if VASP tetramers are required for anti-capping activity, we tested a mutant form of VASP lacking the

**Fig. 4.** Profilin enhances actin polymerization in the presence of VASP and CP. A, profilin increases VASP anti-capping in a dose-dependent manner. Plotted is the time course of actin polymerization in the presence of 1 μM actin, 4 nM CP, 0.2 nM SAS, 25 nM VASP, and varying concentrations of profilin as indicated: none, blue; 1 μM, light blue; 5 μM, orange; 7.5 μM, green; 10 μM, pink; 15 nM, violet. The reaction labeled SAS alone (black) contained no CP, profilin, or VASP. B, dependence of the initial rate of actin polymerization in the presence of CP and VASP on profilin. The initial rates were calculated from a linear fit to the first 60 s of each time course in panel A and normalized to the initial rate obtained in the reaction with SAS alone. C, profilin does not promote spontaneous actin polymerization in the presence of VASP. Plotted is the actin polymer concentration versus time in reactions containing 1 μM actin without (black) and with VASP (25 nM, blue; 50 nM, red) or VASP and 10 μM profilin (25 nM, light blue; 50 nM, orange) as indicated. D, binding of G-actin and the proline-rich region of VASP is required for profilin to enhance actin polymerization in the presence of VASP and CP. Plotted is the time course for reactions containing 2 μM actin, 0.2 nM SAS, 4 nM CP, with 97 nM VASP (violet), and either 10 μM profilin (orange) or mutant profilin (R88E, blue; Y6D, green) as indicated. The reaction depicted in light blue contained 10 μM profilin and no VASP. E, profilin decreases actin assembly in the presence of CP and EVH2. Plotted is the time course for actin polymerization in reactions containing 1 μM actin, 0.2 nM SAS, 10 nM EVH2 (green), or 10 nM EVH2 and 10 μM profilin (blue) as indicated. F, profilin and VASP increase the rate of actin assembly from SAS. Plotted is the time course for actin polymerization in reactions containing 1 μM actin, 0.2 nM SAS with 25 nM VASP (red), 10 μM profilin (green), or 25 nM VASP and 10 μM profilin (blue) as indicated.
indicated. Plotted is the fluorescence of pyrenyl-actin PKA-treated VASP proteins. Reactions were carried out in the presence and absence of 10 nM CP, 0.2 nM SAS, 2 μM actin, and no VASP (red), 50 nM mock-treated VASP/VASP triple mutant (blue) or PKA-treated VASP/VASP triple mutant (orange), without and with 10 μM profilin (mock-VASP/VASP triple mutant + profilin, violet; PKA-VASP/VASP triple mutant + profilin, green) as indicated. Plotted is the fluorescence of pyrenyl-actin versus time.

**TABLE I**

Effect of phosphorylation by PKA on anti-capping activity of wild-type VASP and VASP mutant proteins

| VASP protein         | Mock PKA-treated | 10 μM Profilin + Mock PKA-treated | 10 μM Profilin + PKA-treated |
|----------------------|------------------|-----------------------------------|-----------------------------|
| Wild-type VASP       | 1.1              | 0.6                               | 1.5                         |
| VASP-S153A           | 1.2              | 0.6                               | 1.8                         |
| VASP-S235A           | 1.1              | 0.7                               | 1.4                         |
| VASP-T274A           | 1.2              | 0.7                               | 1.8                         |
| VASP-S153A,S235A     | 1.4              | 1.1                               | 1.7                         |
| VASP-S153A,T274A     | 1.2              | 0.7                               | 1.6                         |
| VASP-S235A,T274A     | 1.2              | 1.0                               | 1.6                         |
| VASP-S153A,S235A,T274A | 1.2         | 1.0                               | 1.4                         |

EVH1 and EVH2 domains of VASP bind profilin and proline-rich regions of profilin. Profilin binds mainly to G-actin (3), but also interacts with high-affinity sites of S153, S235, and T274 on VASP (8, 14, 46). Phosphorylation of wild-type VASP by PKA in vitro abolished VASP anti-capping activity in the presence and absence of profilin (Fig. 5A, Table I). Anti-capping activity of the VASP EVH2 domain was similarly inhibited upon phosphorylation by PKA (data not shown), suggesting that phosphorylation within EVH2 affects VASP anti-capping activity. To determine which of the three phosphorylation sites in VASP were involved in regulating anti-capping activity, we tested mutant forms of VASP in which the phosphorylation sites were changed, singly, doubly, or triply, to alanine. Mutant VASP proteins were phosphorylated with PKA in vitro and tested in anti-capping assays in the presence and absence of profilin (Table I). All mock-

Phosphorylation Decreases VASP Anti-capping and F-actin Bundling Activities—Vertebrate Ena/VASP proteins are substrates for cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively) (27, 34, 42–44). VASP contains three PKA/PKG phosphorylation sites: Ser-153, conserved among all vertebrate family members and situated near the junction between the EVH1 domain and the central proline-rich core, and two sites in the EVH2 domain, Ser-235 and Thr-274, adjacent to the F-actin binding site (amino acids numbered as in murine VASP). In cells, Ser-153 and Ser-235 are preferred targets for PKK and PKG, respectively, however, all three sites can be phosphorylated by either kinase (45). Phosphorylation at the conserved site common to all Ena/VASP proteins (Ser-153 of VASP) is implicated in promoting Ena/VASP function during cell motility, filopodia formation, and in inhibiting fibrinogen binding by α2β3 integrins (8, 14, 46).
treated, non-phosphorylated mutant VASP proteins retained anti-capping activity similar to wild-type VASP. The triple mutant VASP (S153A,S235A,T274A) in which all three phosphorylation sites were changed to alanine, retained nearly all of its anti-capping activity after treatment with PKA; profilin further enhanced anti-capping by the phosphorylated triplet mutant (Fig. 5B, Table I). The slight inhibition of anti-capping activity by PKA-treated VASP triple mutant may result from phosphorylation in vitro at cryptic sites that are not targets of PKA in vivo.

The anti-capping activities of each single-mutant VASP protein, in which one of three phosphorylation sites was replaced by alanine, were nearly fully inhibited by PKA phosphorylation (Table I). Notably, the anti-capping activity of VASP-S153A, which lacks the conserved phosphorylation site implicated in Ena/VASP activity in vivo, was as sensitive as wild-type VASP to phosphorylation by PKA. Therefore, PKA targets other than Ser-153 in VASP negatively regulate anti-capping activity. Analyses of the double mutant VASP proteins (VASP-S153A,S235A, VASP-S153A,T274A, and VASP-S235A,T274A), in which only one phosphorylation site remained, indicated that phosphorylation at Ser-235 contributed primarily to inhibition of anti-capping activity and to the loss of regulation by profilin (Table I). Taken together, these results indicate that phosphorylation of VASP by PKA within the EVH2 domain negatively regulates VASP anti-capping activity in vitro.

VASP binds F-actin and bundles filaments in vitro (3, 25, 26, 29). We confirmed that VASP binds and bundles filaments in light and electron microscope observations of actin filaments (Figs. 6 and 7). Phosphorylation by PKA is reported to decrease the interaction of VASP with F-actin in pelleting assays (47). To determine if phosphorylation by PKA regulates filament bundling by VASP, we analyzed actin filaments formed in reactions containing VASP before and 5 min after addition of PKA. No actin filament bundles were observed after treatment with PKA, suggesting that phosphorylation promotes dissociation of VASP from actin filaments and loss of filament bundling activity (Fig. 6). VASP-GAB, in which mutations of the G-actin binding motif abolished anti-capping activity, retained actin filament bundling activity (data not shown), indicating that anti-capping activity does not result indirectly from formation of filament bundles that shield filament ends from CP.

The morphology and structural organization of VASP-induced filament bundles was salt dependent. At low salt concentrations (15 and 50 mM KCl) the filament bundles are tightly packed (Fig. 7, A, B, and D) with regular cross-bands apparent that give rise to well-ordered computed diffraction patterns (Fig. 7C). The meridian reflections in the diffraction patterns suggest the existence of a cross-linker; the height of the meridian reflection suggests a stoichiometry of three cross-linkers per filament crossover (~13.5 actin subunits). At higher salt concentrations the filament bundles were loosely packed with many single filaments emanating from the bundles (Fig. 7, E and F, 100 and 150 mM KCl, respectively); no regular cross-bands were apparent and incoherent diffraction patterns suggested loss of internal order.

DISCUSSION

Our biochemical studies provide insight into the mechanism for how Ena/VASP proteins enhance actin polymerization at filament barbed ends. We propose that Ena/VASP proteins bind actin filaments at or near barbed ends in a manner that permits association of actin and profilin-actin with barbed ends but prevents association of barbed end capping proteins. Three observations support this hypothesis. First, VASP anti-capping activity by PKA negatively regulates anti-capping activity in vitro.
activity depended on the concentrations of both CP and VASP; second, VASP decreased the amount of G-actin in steady state solutions of actin and CP; and third, VASP increased the rate of depolymerization of pre-formed actin filaments in the presence of CP. VASP protected barbed ends from other barbed end capping proteins, suggesting that VASP generally restricts access of barbed end-binding proteins to filament ends. VASP also enhanced the rate of filament elongation from SAS, suggesting that VASP anti-capping activity may result, in part, from changes in the rates of actin subunit addition or dissociation at barbed ends. The relative contributions of the effects of VASP on filament elongation and direct competition with CP for barbed ends to anti-capping activity remain to be determined.

The EVH2 domain of VASP or Mena was sufficient for anti-capping activity, and three structural motifs within EVH2 were essential: the G-actin binding motif, the F-actin binding motif, and the tetramerization motif. These structural features suggest a model for how Ena/VASP proteins associate with F-actin in which the GAB motif within EVH2 interacts with the terminal actin subunits at barbed ends. The basic GAB motif may interact with an electronegative patch on the surface of subdomain 1 of the terminal actin subunits, overlapping the region occupied by the actin-capping domain of gelsolin domain 1, and by barbed end-binding toxins (48–50). However, unlike capping proteins that inhibit actin assembly, Ena/VASP proteins promote actin assembly, and may do so via interactions of GAB with terminal actin subunits similar to those of the central actin binding motifs of thymosin β4 (51) and ciboulot (52). Additional interactions via the F-actin binding motif within EVH2 may stabilize the association at barbed ends. The requirement for VASP tetramers for anti-capping activity suggests that Ena/VASP proteins may interact with each protofilament to stably dock at barbed ends.

Profilin further enhanced VASP anti-capping activity via a mechanism that required direct interactions of VASP and profilin-actin. Profilin-actin and VASP could stimulate anti-capping via several possible mechanisms. Binding of profilin-actin to the central proline-rich region could stabilize the interactions of VASP at barbed ends, thereby making VASP more effective in competing with CP. Alternatively, binding of profilin-actin to VASP could increase the local concentration of actin at barbed ends (35). However, because subunit addition at barbed ends is considered to be diffusion limited in dilute solutions (20, 53), locally increased profilin-actin concentrations may not result solely in increased rates of actin assembly. Notably, the FH1–FH2 fragment of some formin proteins, together with profilin, increase the rate for elongation at barbed ends greater than 10-fold (54, 55). VASP may similarly exploit profilin-actin to increase actin polymerization rates.

Some formin family proteins also compete with barbed end-capping proteins, in part, by associating processively with barbed ends as the filaments grow (21, 56, 57). Our data do not support a processive association of VASP at barbed ends during anti-capping because VASP remains bound to filaments and bundles them. Phosphorylation or interactions with other binding partners may regulate the interactions of Ena/VASP proteins with actin filaments in vivo to allow persistent association of VASP with elongating filament ends, but this possibility remains to be investigated. Nonetheless, the ability of profilin-actin to enhance VASP anti-capping activity is similar to that of profilin and some formin proteins to allow rapid elongation at barbed ends, even in the presence of capping proteins.

The structural elements of VASP required for anti-capping activity are similar to those required for Mena to restore normal motility to Ena/VASP-deficient fibroblasts in vivo. Both the G-actin and F-actin binding motifs were essential for normal cell motility (14) and for anti-capping activity in vitro. In contrast, profilin-actin was less critical for Ena/VASP function during whole cell motility, since EVH2 or mutant proteins lacking the proline-rich region restored normal cell motility (14). In cells, regulation of full-length VASP by profilin may be important under conditions where maximal anti-capping activity is required, such as during extension of filopodia. Some of the features required for anti-capping (i.e. FAB and the C-terminal tetramerization motif) were not required for Ena/VASP proteins to stimulate intracellular Listeria movement (32), suggesting that anti-capping activity is not essential for Ena/VASP proteins to support Listeria motility.

VASP binds actin filaments and bundles them, which may have implications for anti-capping activity. VASP was most effective in protecting barbed ends from CP when actin filament mass was low (i.e. at the start of seeded polymerization reactions). Under conditions where filament mass was high, such as in experiments with pre-formed filaments, VASP was less effective in competing with CP, presumably because association of VASP with the sides of filaments would decrease the amount of VASP available to compete with CP for binding filament ends.

Ena/VASP proteins are often situated with subcellular structures having bundled actin filament, such as filopodia and focal adhesions, but the proteins are generally restricted to regions near the ends of filament bundles, rather than along their length. Scaffolding molecules that bind Ena/VASP proteins via interactions with EVH1 motifs also concentrate at the tips of filopodia and lamellipodia and could restrict Ena/VASP proteins to growing filament barbed ends (58). The loosely packed filament bundles formed by Ena/VASP proteins at physiologic ionic conditions appear similar to those observed in nascent filopodia (59) and may provide access to other filament bundling proteins such as fascin as filopodia mature.

VASP anti-capping and filament bundling activities in vitro are inhibited upon phosphorylation by PKA. Phosphorylation at Ser-235, adjacent to the G-actin binding motif, was primarily responsible for loss of anti-capping activity. In contrast, phosphorylation of Ena/VASP proteins by PKA at the phosphorylation site conserved in all vertebrate Ena/VASP proteins (Ser-153 in VASP) did not alter anti-capping activity in vitro. Therefore, phosphorylation at the conserved site likely regulates other functions during cell migration or filopodia formation (8, 14, 32). Interestingly, in platelets, Ser-235 in VASP is the preferred site for phosphorylation by protein kinase G (44), suggesting that distinct Ena/VASP activities may result via different cyclic nucleotide-mediated signaling pathways. Spatially regulated phosphorylation at sites within the EVH2 domain could also influence VASP anti-capping and bundling activities in vivo.

Actin filament-barbed ends play a central role in determining the form and function of cellular structures dependent on actin filaments. The ability of Ena/VASP proteins and profilin-actin to modulate filament elongation at barbed ends and protect barbed ends from capping highlights them as key regulators of actin-based processes that determine cellular form and function. We have focused here on the ability of Ena/VASP proteins and profilin to modulate actin dynamics at barbed ends by preventing barbed end capping. Ena/VASP proteins also interact with several other proteins that influence cellular signaling pathways. Specific recruitment of signaling factors to barbed ends via Ena/VASP proteins may be a direct conduit for transducing extracellular cues toward a dynamic actin cytoskeleton.
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