

How VASP enhances actin-based motility

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The function of vasodilator-stimulated phosphoprotein (VASP) in motility is analyzed using a biomimetic motility assay in which ActA-coated microspheres propel themselves in a medium containing actin, the Arp2/3 complex, and three regulatory proteins in the absence or presence of VASP. Propulsion is linked to cycles of filament barbed end attachment-branching-detachment-growth in which the ActA-activated Arp2/3 complex incorporates at the junctions of branched filaments. VASP increases the velocity of beads. VASP increases branch spacing of filaments

in the actin tail, as it does in lamellipodia in living cells. The effect of VASP on branch spacing of Arp2/3-induced branched actin arrays is opposed to the effect of capping proteins. However, VASP does not compete with capping proteins for binding barbed ends of actin filaments. VASP enhances branched actin polymerization only when ActA is immobilized on beads or on *Listeria*. VASP increases the rate of dissociation of the branch junction from immobilized ActA, which is the rate-limiting step in the catalytic cycle of site-directed filament branching.

Introduction

Vasodilator-stimulated phosphoprotein (VASP), originally identified as the major substrate of cGMP- and cAMP-dependent kinases in platelets (Waldmann et al., 1987), is a member of the Ena/VASP family (for reviews see Bear et al., 2001; Reinhard et al., 2001; Kwiatkowski et al., 2003). Ena/VASP proteins play an important role in cell adhesion and migration, and are involved in the regulation of actin-based motility (Gertler et al., 1996; Holt et al., 1998; Bear et al., 2000). However, their detailed mechanism of action remains elusive. VASP is localized at focal adhesions (Gertler et al., 1996; Holt et al., 1998) and at the leading edge and filopodia tips of motile cells (Lanier et al., 1999; Rottner et al., 1999; Svitkina et al., 2003). This double localization may reflect VASP versatility in its interactions with different partners, and may be responsible for the complex function of VASP in cell movement.

Evidence for a role of Ena/VASP proteins in motility came from analysis of the effects of VASP deletion/over-expression on the motile behavior of living cells (Rottner et al., 1999; Bear et al., 2000, 2002; Garcia Arguinzonis et al., 2002; Han et al., 2002) and on actin-based propulsion of

Listeria (Chakraborty et al., 1995; Smith et al., 1996; Laurent et al., 1999; Skoble et al., 2001). Actin-based motility is mediated by site-directed activation of the Arp2/3 complex by the protein ActA (in *Listeria*) or by the proteins of the Wiskott-Aldrich syndrome protein (WASP) family (in lamellipodia and filopodia extension), which catalyze production of new filaments by barbed end branching, thus generating propulsive or protrusive forces (Pantaloni et al., 2001). VASP and other members of the family, like the murine homologue of Ena (Mena) or Evl, greatly enhance the motility of *Listeria* in living cells (Smith et al., 1996) and in cell extracts (Laurent et al., 1999), as well as in a reconstituted motility medium containing only five pure proteins (Loisel et al., 1999), indicating that the function of VASP in *Listeria* motility can be explained by its interaction with a restricted number of protein partners. Ena/VASP proteins are composed of three domains; an NH₂-terminal Ena/VASP homology (EVH) 1 domain (Ball et al., 2002; Renfranz and Beckerle, 2002), a central proline-rich domain, and a COOH-terminal EVH2 domain that binds F-actin with a 1:1 stoichiometry and low affinity (c 2 μM; Laurent et al., 1999), explaining its nucleating effect in actin polymerization (Bachmann et al., 1999; Huttelmaier et al., 1999; Laurent et al., 1999; Walders-Harbeck et al., 2002). The function of

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Abbreviations used in this paper: Mena, murine homologue of Ena; VASP, vasodilator-stimulated phosphoprotein; WASP, Wiskott-Aldrich syndrome protein.

VASP in *Listeria* movement requires binding of the EVH1 domain to consensus FPPPP proline-rich repeats in ActA (Niebuhr et al., 1997) as well as its EVH2 domain (Laurent et al., 1999). How the EVH1 domain of VASP targets the protein to the leading edge or to focal adhesions is not known; however, VASP has been reported to interact with vinculin (Brindle et al., 1996), zyxin (Drees et al., 2000), and WASP (Castellano et al., 2001). Accordingly, VASP enhances the motility of WASP-coated beads (Castellano et al., 2001).

The effect of VASP on motility of living cells is less clear than its effect on *Listeria* propulsion. The amount of VASP at the leading edge correlates positively with the rate of extension of the lamellipodium (Rottner et al., 1999). VASP is required for filopodia extension and for efficient chemotaxis of *Dictyostelium discoideum* (Han et al., 2002). The absence of VASP results in impaired motility and reorientation of cells (Garcia Arguinzonis et al., 2002). On the other hand, in another report, cells in which both VASP and Mena have been knocked out show an increased motility and lamellipodia display a highly branched actin array, whereas cells overexpressing Ena/VASP protrude faster and lamellipodia display a sparsely branched actin array (Bear et al., 2002). To reconcile all the data, it was proposed that VASP inhibited barbed end capping of filaments, thus enhancing the instantaneous rate of protrusion, yet not allowing the persistent protrusion that is required for productive locomotion (Bear et al., 2002). Although the inhibition of barbed end capping by VASP has not been confirmed (Boujemaa-Paterski et al., 2001), it is remarkable that a highly branched actin array is generated by an excess of capping proteins in a reconstituted motility assay of neural WASP-coated beads (Wiesner et al., 2003). In other words, either the absence of VASP or the high activity of capping proteins promote the same morphology of highly branched actin arrays in motile processes. Vice versa, a high level of VASP or a low activity of capping proteins correlate with sparsely branched arrays.

To understand the mechanism by which VASP can control the velocity and the morphology of branched actin networks, we have combined a biochemical approach of the effect of VASP on site-directed polymerization of actin into branched filaments and a biophysical approach of its effects on actin-based movement of ActA-coated beads in a chemically controlled motility medium. We propose that VASP enhances motility by increasing the rate of dissociation of the branched junction, which is the rate-limiting step in the cycle of filament attachment-branching-detachment catalyzed by immobilized ActA at the surface of the beads. Consistently, modeling of the process of assembly of branched filaments at the surface of ActA-coated beads within this scheme reconstitutes the observed experimental curves.

Results

Characterization of ActA-coated beads

2- μm -diam polystyrene beads were coated with ActA using a range of concentrations from 0 to 5 μM . The density of ActA at the surface of the beads (Fig. 1) saturated at a value of 1 molecule per 50 nm^2 , corresponding to an average distance of 5–7 nm between the ActA molecules. To compare

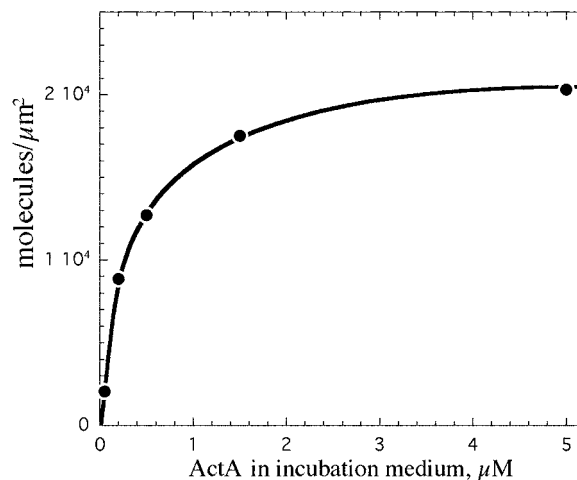


Figure 1. Functionalization of ActA-coated beads. 2- μm -diam carboxylated polystyrene beads (0.25% solids in B buffer) were incubated with ActA at the indicated concentrations for 1 h at 4°C on a Roto-Shake machine, and processed as described in Materials and methods. 20 μl of the suspension of washed beads was denatured and submitted to SDS-PAGE/Western blotting for determination of the amount of bound ActA. Data are accounted for by a standard hyperbolic saturation curve consistent with an equilibrium dissociation constant K_d of 0.3 μM for ActA adsorption to carboxylated beads.

the surface density of ActA on the 2- μm -diam beads with the density of ActA at the surface of *Listeria*, an identical number of ActA-saturated beads and *Listeria* bacteria were sedimented, and the amount of ActA in the pellets was determined by Western blotting. An average number of 22,000 molecules of ActA was found present at the surface of a *Listeria* bacterium, and 260,000 molecules of ActA at the surface of a bead. Assuming the bacterium to be an ellipsoid of $0.5 \times 1 \mu\text{m}$, the surface of a bacterium is 1.34 μm^2 , whereas the surface of a 2- μm -diam bead is 12.6 μm^2 . The average ActA surface densities for the bacterium and the beads are 0.016 and 0.02 ± 0.004 molecules per nm^2 (allowing 10% variation in the bead diameter), respectively. In conclusion, the beads are close homologues of *Listeria* regarding the density of filament-branching enzyme bound to the surface.

VASP enhances site-directed assembly of branched filaments

Actin was polymerized at physiological ionic strength (0.1 M KCl and 1 mM MgCl_2 , pH 7.5) in the presence of soluble ActA and Arp2/3 complex. As reported previously, the addition of VASP (up to 150 nM) did not affect the time course of ActA–Arp2/3-induced polymerization of actin in branched filaments (Fig. 2 A; Boujemaa-Paterski et al., 2001). This means that the frequency of branching, which determines the shape of the polymerization curve, is unchanged. Consistently, neither the branching density of filaments nor the debranching rate was affected by VASP (Boujemaa-Paterski et al., 2001).

In striking contrast, when branching of filaments by the Arp2/3 complex was catalyzed by ActA immobilized at the surface of beads, VASP greatly enhanced the rate of polymerization (Fig. 2 B). This effect required the Arp2/3 com-

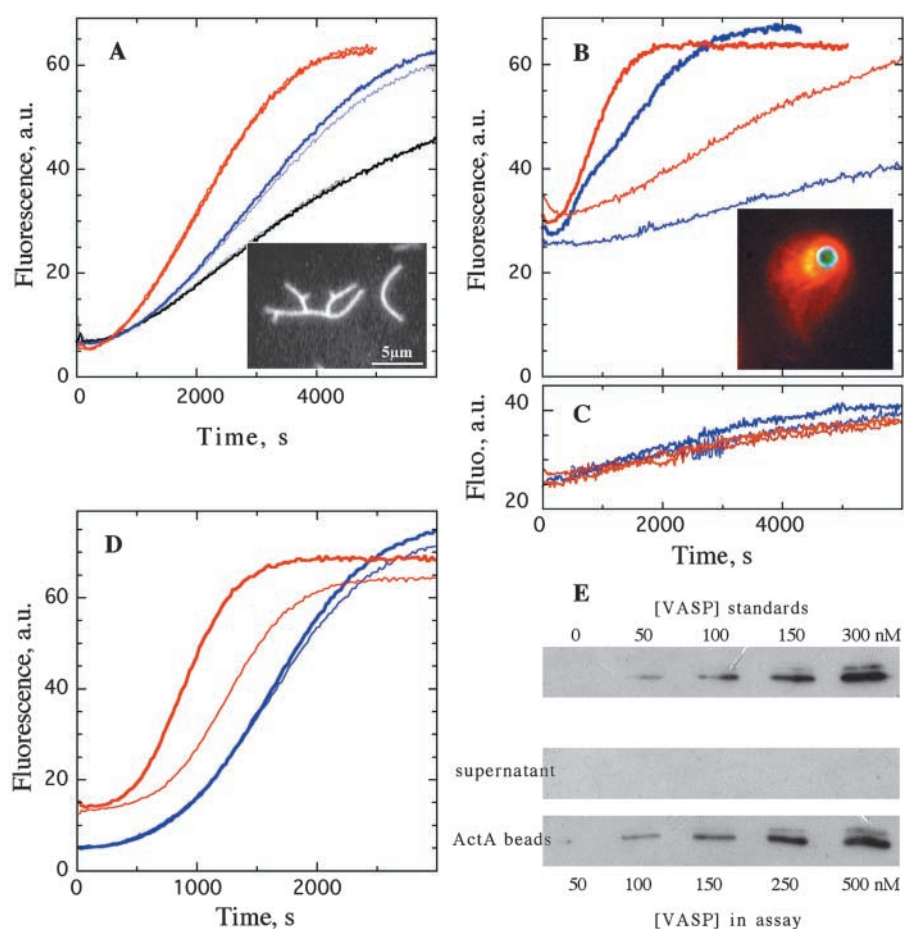


Figure 2. VASP stimulates site-directed assembly of branched filaments by ActA-activated Arp2/3 complex, but does not affect the branching reaction in solution. $3 \mu\text{M}$ actin (10% pyrene labeled) was polymerized in the presence of 50 nM Alexa[®] 488-labeled Arp2/3 complex and either soluble ActA (A) or ActA immobilized on beads (B). Polymerization was performed in the absence (thin curves) or presence (thick curves) of 150 nM VASP. Polymerization was monitored by the increase in pyrene fluorescence. Black curves (A) are controls without ActA, with and without VASP as defined above. The final amount of either soluble or immobilized ActA was the same (2.04 pmol in the blue curves, and 3.24 pmol in the red curves in $120 \mu\text{l}$, in A and B). Inset in A shows branched filaments after rhodamine-phalloidin staining; inset in B shows actin cloud around the $2\text{-}\mu\text{m}$ -diam ActA-coated beads. (C) Same conditions and symbols as in B, except Arp2/3 was not present. (D) *Listeria* bacteria were used as a source of immobilized ActA (red curves); the same amount of soluble ActA as the amount of bacteria-associated ActA was used (blue curves). The high intensity signal measured at time zero in B–D is due to the light scattered by the bead or bacteria suspension. (E) VASP binds to ActA-coated beads in the motility medium. Beads were coated with 2×10^4 molecules ActA/ μm^2 , placed in the

motility medium containing no actin and VASP at the indicated concentrations, sedimented, and resuspended in the original volume. The presence of VASP in the supernatants and bead pellets was immunodetected using an anti-Xpress[™] antibody (Invitrogen).

plex because VASP in itself showed no nucleating effect of actin assembly off ActA-coated beads placed in the actin solution (Fig. 2 C). The effect of VASP was observed in the whole range of ActA surface density. The same effect of VASP was observed when a suspension of *Listeria* bacteria was used instead of ActA-derivatized beads to initiate actin assembly with the Arp2/3 complex (Fig. 2 D). Sedimentation of ActA-coated beads showed that at least 90% of the VASP protein added to the solution was bound to immobilized ActA under those conditions (Fig. 2 E), as is routinely observed for *Listeria* in cell extracts. Thus, when it is adsorbed on beads, ActA retains the same functional properties regarding VASP and Arp2/3 binding as when it is expressed at the surface of *Listeria*. The effect of VASP on site-directed assembly of branched filaments was detectable at concentrations as low as 10 nM and was maximum at 100 nM .

VASP is known to display electrostatic interactions with G-actin that lead to a strong nucleating effect at low ionic strength. In G buffer, VASP readily induces polymerization of actin in F-actin bundles (Huttelmaier et al., 1999; Laurent et al., 1999). This effect vanishes upon increasing ionic strength. At physiological ionic strength (0.1 M KCl and 1 mM MgCl_2), the nucleating activity of VASP was detectable at $0.25 \mu\text{M}$ and increased upon increasing VASP concentrations in the micromolar range (Boujemaa-Paterski et al., 2001; present paper). Consistently, at the

lower physiological ionic strength (10 mM KCl and 2 mM MgCl_2) used by Skoble et al. (2001), the nucleating effect was visible at 40 nM VASP. Under physiological ionic conditions, the one order of magnitude lower concentration range at which VASP affects motility and site-directed actin polymerization off bead-bound ActA suggests that the motile function of VASP is mediated solely by a high affinity interaction with the proteins that are involved in the formation of branched filaments (i.e., ActA, actin, and the Arp2/3 complex).

VASP is required for sustained, fast movement of ActA-coated beads

Actin-based propulsion of ActA-coated beads was monitored in the reconstituted motility medium, in the absence and presence of VASP, using beads with different ActA surface densities. In the presence of VASP, beads moved in a stationary regime at $4\text{--}5 \mu\text{m}/\text{min}$ for over 2 h. In agreement with a preliminary observation (Boujemaa-Paterski et al., 2001), in the absence of VASP, beads initiated actin polymerization at their surface, and after the break of symmetry of the actin “cloud,” they moved at rates of $1\text{--}2 \mu\text{m}/\text{min}$. Movement was often irregular with periodic velocity, leading to “striped” comet tails. In addition, movement stopped after some time, beads lost their tails, and movement never resumed (Fig. 3 A). Using rhodamine-labeled

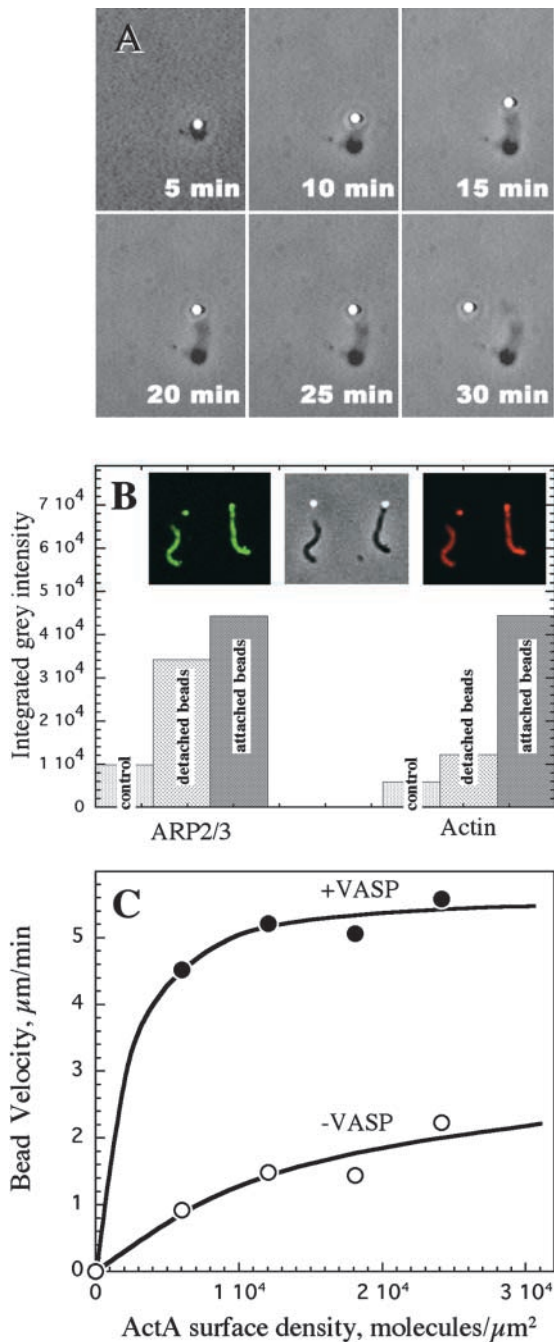


Figure 3. VASP enhances and maintains sustained movement of ActA-coated beads. (A) Time-lapse phase-contrast microscopy images of an ActA-coated bead undergoing actin-based movement in the reconstituted motility medium without VASP. Note the gradual decrease in actin density in the tail leading to arrest of movement and loss of the connection between the bead and the tail. (B) Quantitation of Arp2/3 and actin on 2-µm-diam beads that are attached to actin tails and beads that have lost their actin tails. Top: phase-contrast and fluorescence images of 9% rhodamine-labeled actin and 100% Alexa[®] 488-labeled Arp2/3 on ActA-coated beads with attached and detached actin tails. Bottom: diagram of the integrated fluorescence intensities of actin and Arp2/3 at the surface of detached and attached beads in the motility medium. The fluorescence intensities of actin and Arp2/3 on beads that have an actin tail (attached beads) are normalized to the same level for convenience. Controls represent the amount of actin (Arp2/3) on beads placed in the motility medium without Arp2/3 (Actin). (C) VASP increases the

Table I. Effect of VASP on the motility behavior of beads functionalized with different surface densities of ActA

	Surface density	Beads that form tails	Average time period before movement stops
	molecules/100 nm ²	%	min
- VASP	0.85	10	10–15
+ VASP	0.85	97	60–90
- VASP	1.36	90	40
+ VASP	1.36	98	>120
- VASP	1.85	94	45
+ VASP	1.85	100	>120
- VASP	2.12	97	60–80
+ VASP	2.12	100	>120

Movement of beads was recorded until the actin tails detached from the beads. The time at which 50% of the beads ($n = 200$) had lost their tails was noted.

actin and Alexa[®] 488-labeled Arp2/3 complex in the motility medium revealed that when the beads stopped, both actin and the Arp2/3 complex were still bound at their surface (Fig. 3 B). The amounts of bound actin and Arp2/3 were higher than those recorded when the beads were incubated in the motility medium deprived from either actin (Arp2/3 control) or the Arp2/3 complex (actin control), suggesting that no long filaments, but possibly branched junctions containing actin and Arp2/3, are attached to ActA at the surface of arrested beads. The period during which beads were motile increased with the surface density of ActA from a few minutes to one hour and a half for ActA-saturated beads (Table I). If new beads were added to the “used” medium after arrest of movement of the first beads, they behaved identically (i.e., they moved for the same period of time as the first beads and stopped). If beads that had stopped moving were washed and transferred into either the used or a fresh medium, they moved again for the same period of time as in the first assay and stopped. If beads that had stopped moving were supplemented with 150 nM VASP, they immediately started to move again at a three- to fourfold faster rate (4–5 µm/min) than in the absence of VASP (Fig. 3 C), and movement was sustained for at least 2 h. The effect of VASP on velocity was recorded in a wide range of ActA surface densities, suggesting that VASP enhances a reaction that controls velocity. Overall, these results demonstrate that VASP plays a role in maintaining the persistence of movement, which is linked to the cycle of attachment-branching-detachment-growth of filament barbed ends to the ActA-actin-Arp2/3 complex at the surface of the beads. The arrest of movement was not due to the permanent inactivation of ActA, nor to the exhaustion of some factor from the medium. Rather, the dependence of the duration of sustained movement on the surface density of ActA suggests that some “poisoning” of immobilized ActA develops gradually and prevents the persistence of actin-based movement, and this poisoning is relieved by VASP.

velocity of ActA-coated beads. Average rates of propulsion were measured in the standard motility medium for 2-µm-diam beads, at the indicated ActA surface density, in the absence (open symbols) and presence (closed symbols) of 150 nM VASP.

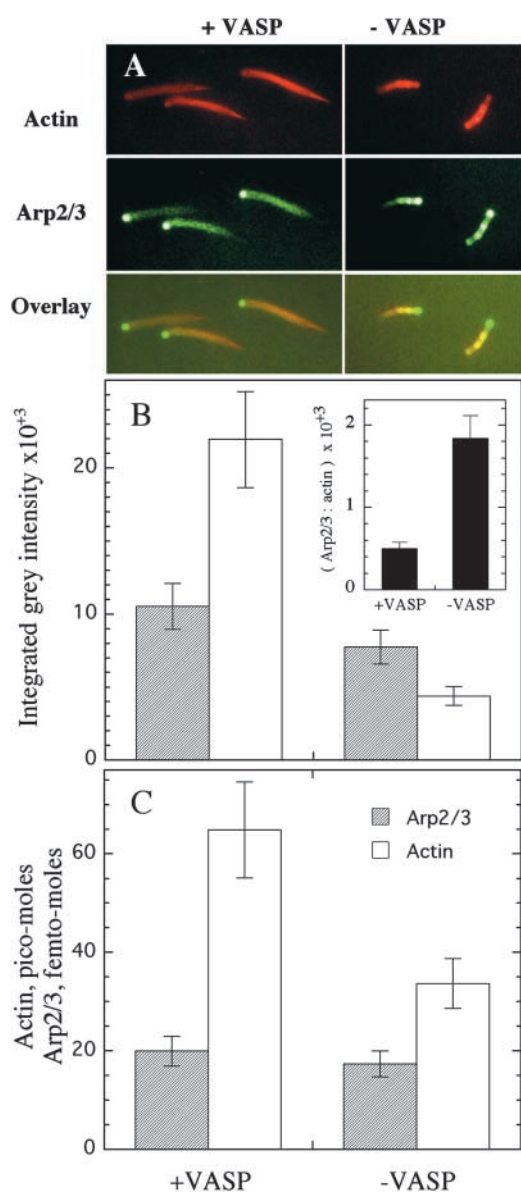


Figure 4. VASP decreases the density of filament branching in the actin tails of ActA-coated beads moving in the reconstituted motility medium. (A) Typical fluorescence images of actin and Arp2/3 complex in the tails. The movement of ActA-coated beads was monitored in the standard motility medium containing 3% rhodamine-labeled actin and 100% Alexa[®] 488-labeled Arp2/3 in the absence or presence of 150 nM VASP. The same settings of the camera were used for rhodamine and Alexa[®] fluorescences. (B) Diagram showing the fluorescence-integrated intensities of rhodamine-actin (open bars) and Alexa[®] 488-Arp2/3 (gray hatched bars) in the actin tails. Inset: Arp2/3/actin ratio, in the presence and absence of VASP. (C) Diagram showing the amounts of actin and Arp2/3 in actin tails derived from a sedimentation assay. The Arp2/3:actin ratio derived from this assay is lower than in B because the measurement includes the Arp2/3 bound to the surface of the bead in addition to the Arp2/3 in the tail.

VASP controls the frequency of filament branching

The motility of ActA-saturated beads was monitored in the reconstituted motility medium containing 3% rhodamine-labeled actin and 100% Alexa[®] 488-labeled Arp2/3 complex, in the absence and presence of 150 nM VASP. The fluorescence intensities of actin and Arp2/3 in the actin tails

were integrated. Data summarized in Fig. 4 show that in the presence of VASP, the actin tails displayed a fourfold higher actin density than in the absence of VASP, but contained only a 20% higher amount of Arp2/3 complex, indicating that actin polymerized more efficiently and filaments were less densely branched in the presence than in the absence of VASP. The Arp2/3:actin ratio, which represents the branching density of the filament array in the actin tail (the reciprocal of the branch spacing), decreased 3.6-fold by addition of VASP. The result was confirmed by sedimenting the beads with their actin tails, solubilizing proteins with 4 M urea, and measuring rhodamine and Alexa[®] 488 fluorescences in the supernatants of the beads.

Remarkably, the effect of VASP on motility and structure of the tail of ActA-coated beads in a motility assay of defined composition is identical to the effect of VASP on motility in living cells, in which the target of VASP is not known. Highly branched actin arrays have actually been observed in lamellipodia of (Mena^{-/-}, VASP^{-/-}) cells, whereas over-expression of Mena/VASP leads to sparsely branched lamellipodial actin arrays and faster protrusion rate (Bear et al., 2002).

Evidence for less densely branched actin filaments upon VASP addition when actin was polymerized with soluble ActA and Arp2/3 has been reported previously (Skoble et al., 2001). This result, which seemingly contrasts with the present data showing no change in branch density upon addition of VASP to soluble ActA, actin, and Arp2/3 (Fig. 2 A), is tentatively explained by the fact that at the low ionic strength used by Skoble and colleagues, VASP displays an intrinsic nucleating effect, and by itself generates unbranched filaments that form together with the ActA-Arp2/3-induced branched filaments (Skoble et al., 2001). The additional formation of unbranched filaments is likely to be responsible for the decrease in the average branching density. Due to the simultaneous activities of VASP under those conditions, it is difficult to definitely conclude that VASP affects the branching density of filaments polymerized via soluble ActA-Arp2/3.

VASP does not directly compete with capping proteins for binding barbed ends of filaments

In a recent work, we showed that the density of filament branching increases with the concentration of capping protein in the motility medium (Wiesner et al., 2003). This result is supportive of models for actin-based motility in which the balance between barbed end branching and capping is an essential ingredient (Carlsson, 2001, 2003). The effect of VASP on the morphology of actin arrays formed in the motility medium is opposite to the effect of capping proteins. It has recently been proposed that VASP could interact with barbed ends, thus preventing them from being capped and promoting barbed end growth (Bear et al., 2002). To test this possibility, actin was polymerized with ActA and the Arp2/3 complex, with and without VASP and in the presence or absence of capping protein (Fig. 5 A). No inhibition of capping by VASP was recorded, in agreement with previous assays (Boujemaa-Paterski et al., 2001). Identical results were obtained with capping protein and CapG.

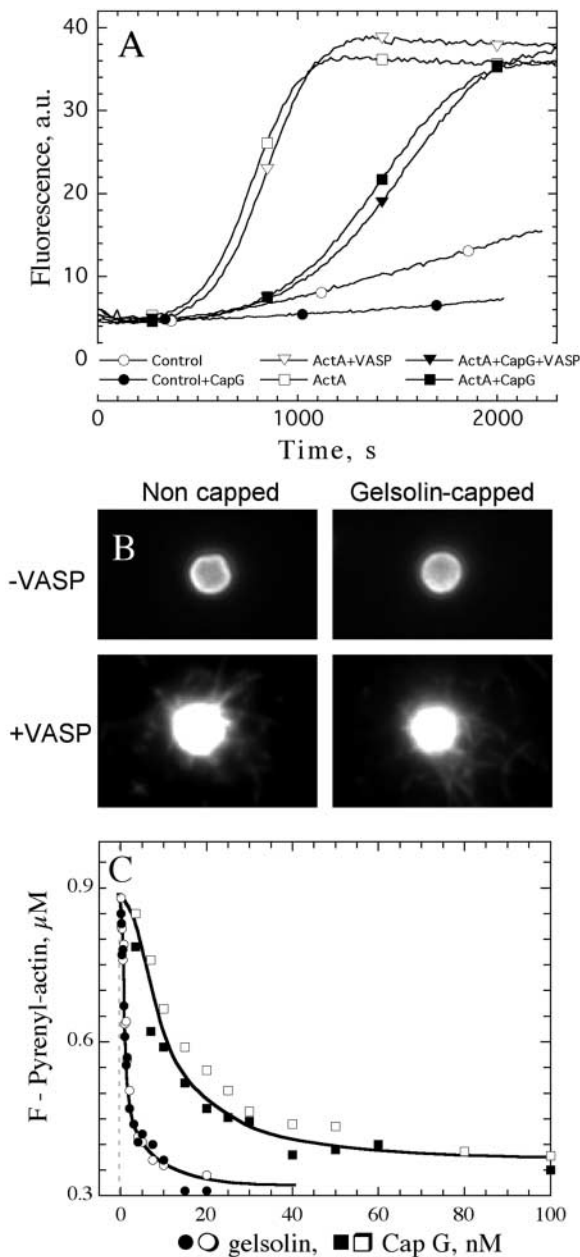


Figure 5. VASP does not compete with capping proteins for binding filament barbed ends. (A) VASP does not prevent the inhibition of barbed end growth by capping protein. Actin was polymerized at $2.5 \mu\text{M}$ with 25 nM Arp2/3, in the absence (open symbols) and presence (closed symbols) of capping protein, without (circles) and with (squares and triangles) $0.2 \mu\text{M}$ ActA, in the absence (squares) and presence (triangles) of 150 nM VASP. (B) VASP-coated beads bind gelsolin-capped or noncapped filaments identically. $5 \mu\text{M}$ actin was polymerized overnight in the absence or presence of 100 nM gelsolin. Filaments were stabilized with 1 M equivalent rhodamine-phalloidin. ActA-coated beads were incubated with $2 \mu\text{M}$ VASP for 1 h at 4°C , washed three times with F buffer, and incubated with noncapped or gelsolin-capped rhodamine-phalloidin-stabilized filaments at 20°C for 10 min . $2\text{-}\mu\text{m}$ -diam beads were washed two times in F buffer, and samples were observed using a microscope (AX; Olympus) with a $100\times$ objective. Images were collected with a CCD camera (Orca II; Hamamatsu) and acquired using MetaMorph® software (Universal Imaging Corp.). Identical results were obtained when filaments assembled from rhodamine-labeled actin and stabilized by unlabeled phalloidin were either not capped or capped by gelsolin before being incubated with ActA–VASP-coated beads.

When ActA beads were coated with VASP, they bound capped and noncapped filaments identically (Fig. 5 B), consistent with VASP binding to the sides of filaments (Bachmann et al., 1999; Huttelmaier et al., 1999; Laurent et al., 1999; Walders-Harbeck et al., 2002). For reasons that we cannot explain, this result is different from the one obtained by Bear et al. (2002), who used FPPPP-derivatized beads instead of ActA-coated beads to immobilize VASP, and found that capped filaments did not bind to immobilized VASP. It is known that VASP binds to the FPPPP repeats in ActA, hence the tethering and the orientation of VASP are expected to be similar, if not identical, when bound to immobilized ActA or FPPPP peptide. However, the two assays may differ in other ways (e.g., the surface density and microenvironment of VASP might be different). Therefore, the difference between our data and the data of Bear et al. (2002) regarding this issue deserves further investigation.

To further examine the competition between capping proteins and VASP for barbed end binding, the effect of VASP on the shift in critical concentration linked to barbed end capping was measured. Addition of increasing amounts of either gelsolin or CapG to $1 \mu\text{M}$ Mg–F-actin caused partial depolymerization of actin consistent with the increase in critical concentration from 0.1 to $0.6 \mu\text{M}$, reflecting binding of the capping protein to barbed ends. Superimposable data points were obtained in the presence or absence of VASP, indicating that VASP does not inhibit barbed end capping by capping proteins (Fig. 5 C).

The measurements of inhibition of barbed end growth off spectrin–actin seeds by capping proteins provides another evaluation of the capping activity, from which the affinity of capping protein for barbed ends can be derived. We confirm that addition of VASP in the range of $0\text{--}3 \mu\text{M}$ reversed the effect of capping proteins on seeded barbed end growth, causing an increase in growth rate as previously reported (Bear et al., 2002). However, a similar VASP-induced increase in rate of barbed end growth was observed in the absence of capping protein, at the same actin concentration ($2 \mu\text{M}$), due to the nucleating effect of VASP, which increases with VASP concentration and is detected at a concentration of $0.25 \mu\text{M}$ and higher, in the early time course of growth when the measurements of initial growth rate are performed.

It is noteworthy that the interplay between the nucleating effect of VASP and the capping of barbed ends by capping proteins generates a complex behavior as follows. When VASP is present in a seeded nucleation assay, it does not merely create a given number of new filaments that add up to the seeds at time zero, but it also constantly generates new filaments in the early time course of the growth assay via interaction between G-actin and VASP. Nucleation slows down as the G-actin concentration in the solution decreases due to barbed end growth. As a result, VASP-induced nucle-

(C) VASP does not affect the shift in critical concentration upon addition of gelsolin or CapG to F-actin. A solution of $1 \mu\text{M}$ F-actin (10% pyrenyl labeled) was polymerized in the presence of 0.2 mM CaCl_2 , 0.2 mM ATP, 0.1 M KCl, and 2 mM MgCl_2 in the presence (closed symbols) or absence (open symbols) of 150 nM VASP. Increasing amounts of gelsolin (circles) or CapG (squares) were added to each sample. Pyrene fluorescence was monitored after 18 h .

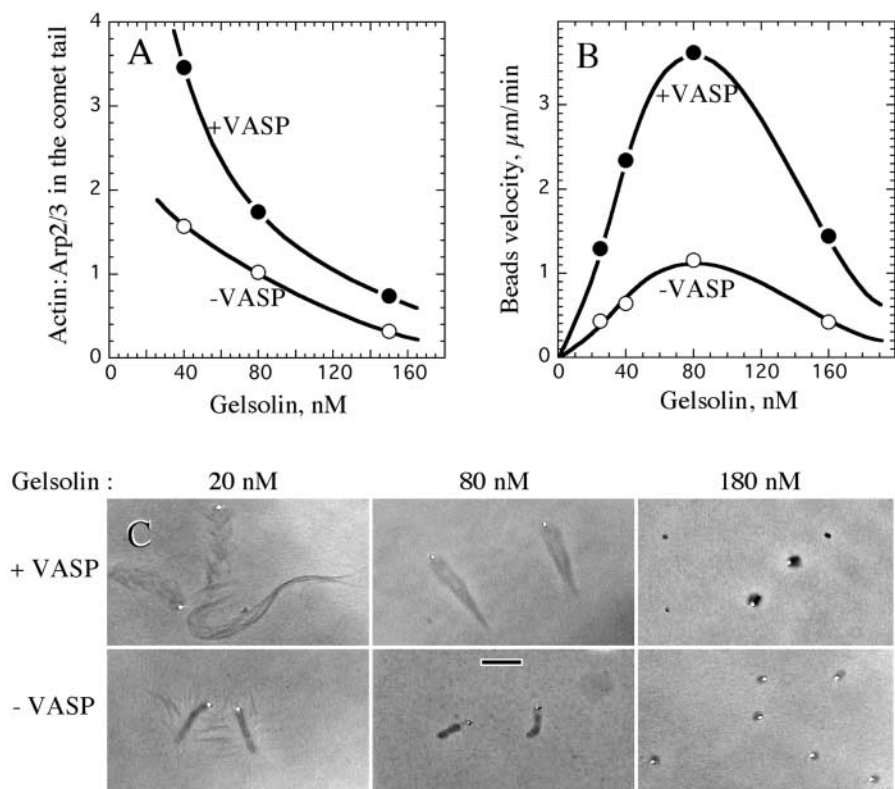


Figure 6. VASP decreases the branching density of actin arrays and increases the velocity of beads in a manner that is not affected by gelsolin. (A) The effect of VASP on the actin:Arp2/3 ratio in tails is not affected by gelsolin. The actin:Arp2/3 ratio in the actin tails was determined at the indicated gelsolin concentrations, in the absence (open symbols) and presence (closed symbols) of 150 nM VASP. The higher actin:Arp2/3 ratio in the presence of VASP demonstrates that VASP decreases the branching density. (B) The effect of VASP on bead velocity is not affected by gelsolin. Average velocities were measured at the indicated concentrations of gelsolin, in the absence (open symbols) and presence (closed symbols) of 150 nM VASP. Note that the two curves correspond to a 3.2-homothetic factor. (C) Typical phase-contrast images of actin tails observed in the presence of VASP (top) and absence of VASP (bottom), at the indicated concentrations of gelsolin. Comet tails display a “fishbone” pattern at low gelsolin concentration, as reported elsewhere (Pantaloni et al., 2000; Wiesner et al., 2003), and they show a higher phase-contrast in the absence of VASP. Bar, 10 μm .

ation is more efficient when a capping protein is present because the concentration of G-actin decreases more slowly. The nucleation of free barbed ends and the capping of these barbed ends are taking place as two independent simultaneous processes. As a result, in the presence of VASP, as the concentration of capper is varied, rate measurements are not done at a constant number of ends, as they must be to derive a relevant binding curve for capping protein; (1) at a given concentration of capper, more ends (capped and non-capped) are present in the presence of VASP than in its absence; and (2) at a given concentration of VASP, the total concentration of ends present in the time interval required for rate measurement increases with the concentration of capper. In other words, the inhibition of barbed end growth by capping proteins is apparently more energetically costly (a higher amount of capper is needed to reach a rate twofold lower than the growth rate measured in the absence of capper) in the presence than in the absence of VASP, even though no direct competition between VASP and capping protein takes place at the barbed ends. These kinetic considerations explain the apparent competition between capping proteins and VASP in seeded growth assays.

To further examine whether the opposite effects of VASP and capping proteins on motility and on the branching density of actin arrays are mediated by competitive direct binding of VASP and capping proteins to barbed ends, the motility of ActA-coated beads was recorded with and without VASP, at different concentrations of gelsolin. The density of actin and Arp2/3 in the tails and the velocities were measured (Fig. 6). At all concentrations of gelsolin, the velocity was increased 3.3- to 3.8-fold by addition of 150 nM VASP. The increase in the actin:Arp2/3 ratio in the tails upon add-

ing 150 nM VASP remained identical as well upon increasing the concentration of gelsolin by fourfold. If VASP competed with capping proteins for binding to barbed ends, a much weaker effect of 150 nM VASP on the velocity or the density of branches would have been measured at high concentration of capping protein. In conclusion, although VASP and capping proteins have antagonistic effects on the motility phenotype, these effects are elicited through different molecular mechanisms.

In conclusion, the effect of VASP on actin-based motility is linked to the stimulation of ActA–Arp2/3-induced site-directed polymerization of actin and to the modulation of the branching density of this network.

Model: VASP regulates the cycle of filament branching

Biochemical analyses (Pantaloni et al., 2000, 2001; Falet et al., 2002), computational simulations (Carlsson, 2001, 2003; Mogilner and Oster, 2003), and chemically controlled motility assays (Wiesner et al., 2003) comfort the view that actin-based movement is generated by site-directed catalytic cycles of filament attachment/detachment coupled to barbed end branching and transient growth, arrested by filament capping. In a stationary regime of movement, some filaments are attached to the particle, engaged in the process of branch formation, and others are detached and growing. Attaching of filaments correlates with the association of a barbed end with the immobilized ternary G-actin–ActA–Arp2/3 complex. Detachment correlates with the dissociation of the Arp2/3-containing branch junction from immobilized ActA, allowing growth of mother and daughter filaments. Growth of the filaments is either arrested by capping of free barbed ends at some distance from the bead, or perpet-

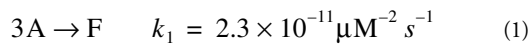
uated by another cycle of branching if barbed ends associate with immobilized ActA. Like the release of the product in an enzymatic catalytic cycle, detachment of the branched junction kinetically limits the catalytic turnover of ActA.

We propose that when ActA is bound to beads, VASP both enhances actin-based movement and decreases the density of filament branching by modifying the kinetics of branched polymerization. In increasing the rate of detachment of the branched junction from immobilized ActA, which is the rate-limiting step in the cycle of filament branching, VASP decreases the fraction of attached filaments that is responsible for the internal friction force opposing actin-based propulsion generated by growing, detached filaments. As a result, filaments grow more efficiently after incorporation of Arp2/3 at the branch, and the distance between the branch junctions (the branch spacing) increases. In the absence of VASP, filaments remain attached for a longer period of time, hence, a greater fraction of the population of filaments are attached, generating a high friction, promoting slow growth of the detached filaments, and increasing the frequency of branching.

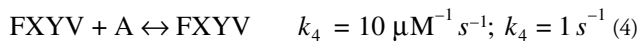
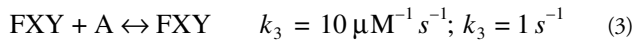
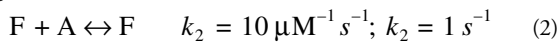
Computer simulation of the polymerization curves generated according to this model was performed using the KIN-SIM software. The following scheme and values of the rate constants were used to obtain the best adjustment of the calculated curves to the data (Fig. 7).

When Arp2/3 is activated by free ActA in solution, the following elementary reactions take place, in which A is G-actin, F is the number concentration of filaments, X is ActA, Y is the Arp2/3 complex, and V is VASP.

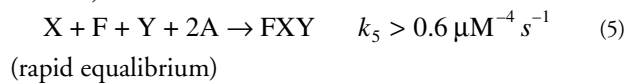
Nucleation



Elongation



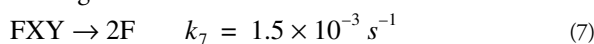
Branch junction formation



VASP binding

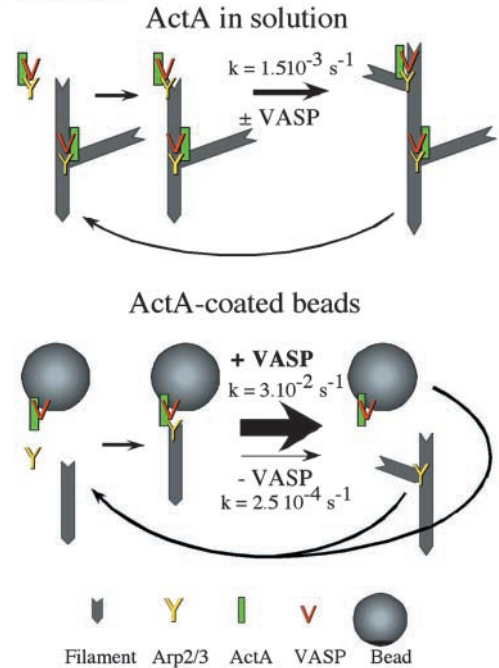


Branching \pm VASP



When Arp2/3 is activated by bead-bound ActA, Eqs. 1–6 are unchanged, but the branching process (Eq. 7) is now linked to detachment of the branch junction from immobilized ActA (Eq. 8) that slows down the reaction. The value of this rate constant is greatly increased by VASP bound to ActA (Eq. 9), as described below.

A : Model



B

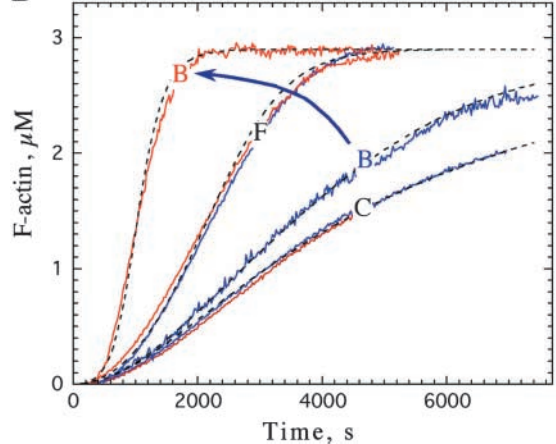
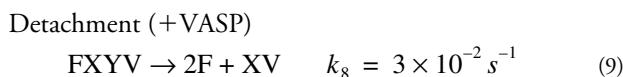
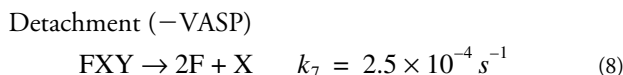


Figure 7. Computer modeling of the effect of VASP on polymerization of actin into branched filaments with soluble or immobilized ActA. (A) Diagram of the model representing the different elementary steps involved in filament branching when ActA is soluble and when ActA is immobilized on beads, and the resulting differences in how VASP affects branched polymerization kinetics in the two situations. When ActA is soluble (top), the availability of activated ActA–Arp2/3 for branching is not limited, and the rate of dissociation of ActA from the branch junction does not play a role in the continued production of branched filaments, hence, the effect of VASP on this reaction cannot be revealed. When ActA is immobilized (bottom), the release of the branch junction from bead-bound ActA is required to allow the branched filament to grow and resume a new branching reaction. VASP accelerates this elementary reaction by two orders of magnitude, thus accelerating the cycle of autocatalytic branching and revealing its function in motility. (B) Fit of the model to experimental polymerization curves of actin in the presence of ActA (soluble or immobilized, 3.24 pmol in 120 μl) and Arp2/3 \pm VASP (data coming from Fig. 2). Noisy curves are experimental, dashed lines are calculated curves using the model and rate parameters given in the text. Blue curves, no VASP; red curves, + VASP; C, control without ActA; B, bead-bound ActA; F, free ActA in solution (blue and red curves superimpose).



Polymerization curves were generated using the above equations, as previously performed for VCA-activated Arp2/3 complex (Pantaloni et al., 2000), with the additional feature that when ActA is bound to the beads, dissociation of the branch junction from immobilized ActA is a rate-limiting step in the overall branching process. This rate-limiting step is strongly accelerated when VASP is bound to ActA.

This model quantitatively accounts for all experimental curves of polymerization into branched filaments obtained with soluble and immobilized ActA, both in the absence and presence of VASP (Fig. 6). The following assumptions were made in adjusting the parameters. The rate constant k_5 for formation of the branch junction (Eq. 5, FXY complex) has a minimum value of $0.6 \mu\text{M}^{-4} \text{ s}^{-1}$, not to become rate limiting. The value of k_7 then was adjusted to fit the experimental curves.

The hypothesis that VASP accelerates the detachment of filament after branching catalyzed by immobilized ActA generates two properties that are consistent with experimental results. First, the effect of VASP on actin polymerization when Arp2/3 is stimulated by immobilized ActA is reconstituted. Second, in the motility assay, VASP-induced rapid detachment of filaments from immobilized ActA leads to an increase in the proportion of detached, growing filaments, thus enhancing velocity and decreasing the frequency of branching, in agreement with observed behavior.

Discussion

The function of VASP in motility so far has been approached in complex cellular environments, using gene deletion and cell transfection with the FPPPP ligand to target endogenous VASP to different cell compartments (Bear et al., 2000, 2002). These experiments showed that in Ena/VASP-deficient cells, lamellipodia extension was slower but more persistent, consistent with more densely branched actin arrays than in Ena/VASP-enriched cells. It was proposed that VASP controlled the geometry of actin arrays by interacting with barbed ends of filaments and shielding them from capping proteins.

Here, we have shown that the effects of VASP on motility and site-directed actin polymerization are reconstituted in a simplified and controlled system that consists of ActA-coated beads undergoing actin-based propulsion in a solution containing actin, Arp2/3 complex, actin-depolymerizing factor, profilin, and a capping protein. VASP enhances bead velocity, maintains the persistence of movement, and decreases the branching density of the actin dendritic array—the three characteristic features of VASP function in cells (Bear et al., 2002). These effects are opposite to those of capping proteins (Wiesner et al., 2003); however, VASP and capping proteins act differently.

VASP enhances motility by increasing the rate of site-directed branching of actin filaments

In the biomimetic motility assay, the increase in velocity due to VASP is a result of an increase in the rate of actin polymerization. Velocity plateaus in the range of 50–150 nM VASP. In a simple polymerization assay that contains only actin and Arp2/3 as soluble proteins, VASP, in the same concentration range, increases the rate of actin polymerization off ActA-coated beads. This effect is not observed when all proteins are soluble, showing that VASP may favor diffusion of the adducts. The activity of immobilized ActA to branch filaments is lower than the one of free ActA. Dissociation of the branched junction is required for growth of the mother and daughter filaments; however, this reaction is not required for filament growth when ActA is free in solution. The rate of dissociation of the branched junction from immobilized ActA controls actin-based motility in determining the proportion of attached (branching) and detached (growing) filaments during movement.

According to the tethered ratchet model (Mogilner and Oster, 2003), detached filaments grow and exert a propulsive force, whereas attached filaments pull and play the role of an internal friction antagonizing the propulsive force (Wiesner et al., 2003). If dissociation of the branched junction is facilitated, the proportion of attached (braking) filaments is lowered, branching density is decreased, and movement is faster. Therefore, it is reasonable to propose that VASP increases the rate constant for dissociation of the branched junction, which is the rate-limiting step in the catalytic cycle of site-directed filament branching.

The effect of VASP on motility is not due to its binding to barbed ends in competition with capping proteins

The present work shows that the effect of VASP on the rate of actin polymerization off ActA beads does not require a capping protein. Polymerization assays show evidence for the absence of competitive binding of VASP and capping proteins to barbed ends. VASP binds to the sides of capped or noncapped filaments identically. The effect of VASP on velocity and on the frequency of filament branching is not affected by capping proteins. In motility assays, VASP is essentially bound to ActA at the surface of beads, while filaments are capped by the soluble capping protein, after growing some distance away from the surface of the bead. Finally, the effect of VASP on motility is optimum at a concentration of VASP (50–150 nM) compatible with *in vivo* concentrations but one order of magnitude lower than the concentration range in which effects were recorded on capping the barbed ends.

These observations do not support the evoked possibility (Bear et al., 2002) that VASP would act by preventing capping of barbed ends by direct competition. Here, we have shown that the apparent competition between capping proteins and VASP for binding to barbed ends was caused by the nucleating activity of VASP. There is a general consensus about the reality of the nucleating activity of VASP (Huttelmaier et al., 1999; Laurent et al., 1999; Bearer et al., 2000; Lambrechts et al., 2000; Skoble et al., 2001); however, data obtained in different laboratories differ quantitatively, and

cannot be directly compared in part because the nucleating activity has not been measured, in all laboratories, under identical conditions of actin and VASP concentrations, actin-bound metal ion, ionic strength, and pH. Nucleation of actin is a cooperative process that is strongly dependent on actin concentration, hence the nucleating effect of VASP, presumably mediated by the stabilization of actin dimers or oligomers, is more prominent at 2 or 4 μM actin (Bear et al., 2002; Skoble et al., 2001; present paper) than at 1 μM actin (Laurent et al., 1999). Second, Mg-actin nucleates more easily than Ca-actin (Carlier et al., 1986), hence, the nucleating effect of VASP is more easily detectable on Mg-actin than on Ca-actin (Lambrechts et al., 2000). Third, nucleation of actin is energetically favored at a lower pH (Zimmerle and Frieden, 1988), hence, the effect of VASP is more easily detectable at pH 7.0 than at pH 8.0. Finally, the nucleating effect of VASP is due to electrostatic interactions and is very dependent on ionic strength (Laurent et al., 1999). VASP nucleates at lower concentration in the presence of 10 mM KCL (Skoble et al., 2001) than at physiological ionic strength (Bear et al., 2002; present paper).

In lamellipodia, VASP may modulate the filament-branching activity of a eukaryotic filament-branching enzyme, homologue of ActA, thus maintaining persistence of actin-based movement

Interestingly, the effects of VASP on the density of filament branching in cells are reproduced in vitro in the ActA-based reconstituted motility system. This strongly suggests that the molecular mechanism underlying the effect of VASP is general, and that in vivo VASP modulates the activity of an immobilized, membrane-bound filament-branching machinery (activator of Arp2/3 complex, functional homologue of ActA) so as to enhance the dissociation of the branched filament and to allow faster growth and protrusion. At apparent variance with this view, two reports emphasize that the molecular requirements for VASP function differ between *Listeria* and lamellipodia (Geese et al., 2002; Loureiro et al., 2002). That different regions of VASP do not play identically important roles in motility of *Listeria* and in motility of living cells may reflect the fact that the strength of the VASP–target interaction is not distributed identically among the different regions of the proteins in the two processes. For instance, the central proline-rich region of VASP might be engaged in a stronger interaction with the ActA machinery than with the corresponding filament-branching machinery in living cells.

Other proteins of the VASP family, namely Mena and Evi, which replace VASP to enhance motility of *Listeria*, are likely to act like VASP in cell motility, increasing the rate of dissociation of the branched filament, each acting on a specific filament-branching enzyme. The identities of these branching enzymes remain unknown. However, in this respect, VASP has been shown to increase the motility of WASP-coated beads (Castellano et al., 2001), and may be acting via a similar mechanism.

The apparently puzzling poisoning of ActA-coated beads that leads to the arrest of movement after a transitory propulsive activity is consistent with the gradual saturation of

bead-bound ActA molecules by branched structures that contain actin and the Arp2/3 complex and do not dissociate fast enough to maintain a sufficient steady-state amount of unliganded ActA, able to react with new filament ends. Because filaments undergo treadmilling in the actin tail, capped filaments depolymerize from their pointed ends and disappear if they are not perpetuated by barbed end branching. Hence, filaments that remain attached to ActA for a long time are borne to shorten, eventually losing connection with the rest of the tail and becoming “dead-end poisoning complexes.” Thus, the perpetuation of the actin array is blocked and the actin tail detaches from the bead. It is very likely that this mechanism is at the heart of the generation of periodic actin tails and oscillatory regime of movement of ActA-coated beads in the absence of VASP (Fig. 2 B), and of “hopping *Listeria*” in vivo and in cell extracts (Lasa et al., 1997; Lauer et al., 2001). The periodic oscillatory regime has also been reconstituted in the motility medium made of pure proteins, using WASP-coated beads at high surface density (Bernheim-Grosswasser et al., 2002).

The regulation of branch spacing by the balance between VASP and capping proteins has important consequences in specifying the different morphologies of actin-based protrusions. Filaments are densely branched in lamellipodia and may be very sparsely branched in filopodia due to the lower abundance of capping protein and/or the higher level of VASP. This hypothesis needs to be challenged by further experimentation.

Materials and methods

Proteins

Actin was purified from rabbit muscle and isolated in the CaATP–G-actin form by gel filtration on Superdex™ 200 columns (Amersham Biosciences) in G buffer (5 mM Tris-Cl⁻, pH 7.5, 1 mM DTT, 0.2 mM ATP, 0.1 mM CaCl₂, and 0.01% NaN₃). Actin was labeled on Cys374 using pyrenyl-iodoacetamide and on lysines using rhodamine-NHS (Wiesner et al., 2003). The Arp2/3 complex was purified from bovine brain (Egile et al., 1999) and labeled with Alexa® 488–maleimide (Molecular Probes, Inc.) as described previously (Wiesner et al., 2003). Recombinant human actin-depolymerizing factor, bovine brain profilin, capping protein, and recombinant human plasma gelsolin were purified as described previously (Loisel et al., 1999). His-tagged human VASP was produced and purified from insect Sf9 cells using the baculovirus expression system (Laurent et al., 1999). His-tagged full-length ActA (ActA[1–584Rhis]) was purified from cultures of *Listeria* strain BAC 093 (*Listeria monocytogenes* L028ΔActA) as described previously (Cicchetti et al., 1999).

Functionalization of beads with ActA

1–2- μm -diam polystyrene microspheres (Polysciences) in a suspension containing 0.25% solids were incubated with ActA at concentrations in the range of 0–5 μM in buffer A (10 mM Hepes, pH 7.8, 0.1 M KCl, 1 mM MgCl₂, 1 mM ATP, and 0.1 mM CaCl₂) for 1 h on ice. 10 mg/ml BSA was added and incubation was continued for 15 min. Beads were washed and stored in buffer A containing 1 mg/ml BSA and could be kept for at least 4 d on ice without any loss in activity. The density of ActA on the bead was determined by SDS-PAGE of a given amount of beads, followed by immunodetection of ActA using anti-ActA–Y21T affinity-purified antibodies against the second proline-rich repeat of ActA (Kocks et al., 1993).

Polymerization assays using ActA-coated beads

Actin (3 μM , 10% pyrenyl-labeled) was polymerized in the presence of 50 nM Arp2/3 complex, with or without 50 nM VASP, and ActA-coated beads representing a final suspension of 0.033% solids (from a 0.2% solids stock suspension). ActA-coated beads containing different surface densities of ActA were used. Control assays were run in which the same amount of soluble ActA was present in the medium instead of being immobilized at the

surface of the beads. Polymerization was monitored by the increase in pyrene fluorescence using a Safas spectrofluorimeter allowing simultaneous recording of up to 10 samples.

Motility assay using ActA-coated beads

The standard motility medium (Loisel et al., 1999; Wiesner et al., 2003) consisted of a solution of 7 μ M F-actin in buffer A, supplemented with 3.5 μ M actin-depolymerizing factor, 2.4 μ M profilin, 80 nM gelsolin, and 100 nM Arp2/3 complex, with or without VASP (50–200 nM as indicated). In addition, the assay contained 5 mg/ml BSA, 0.2% (wt/vol) methyl cellulose (cat. # M-0512, Sigma-Aldrich; yielding a viscosity of 4,000 cP at 2% wt/vol), 7 mM DTT, and 1.5 mM diazo-bicyclo-octane. Changes in the composition of the standard medium are indicated in the text.

Average rates of movement were determined as follows. Synchronous films of up to four selected fields were recorded with a CCD camera (Panasonic) on a microscope (model AX70; Olympus) equipped with a motorized stage (Märzhäuser) using MetaMorph[®] software, version 4.6 (Universal Imaging Corp.) for microscope control and image acquisition. A 20 \times phase objective (NA 0.5) was used. Freely moving beads from at least two different fields were selected. The template recognition-based tracking tool of MetaMorph[®] was used to measure mean velocities. Average mean velocities were calculated for sets of 5–10 beads per assay.

Measurements of branching density in the actin network

The relative densities of actin and Arp2/3 complex in the actin tails of beads moving in the reconstituted motility medium were evaluated using 3% rhodamine-labeled actin and 100% Alexa[®] 488-labeled Arp2/3 complex in the motility medium. These labeling ratios ensured that the same settings of the camera could be used to monitor actin and Arp2/3 fluorescences, and that no overlap of one of the signals on the other occurred (Wiesner et al., 2003). Images of at least eight moving beads were collected using the Olympus microscope with a 40 \times oil objective, appropriate filters for rhodamine and Alexa[®] 488 fluorescences, and a Lhesa analogue video camera. The average rhodamine and Alexa[®] 488 fluorescence intensities were measured in each tail using MetaMorph[®] software.

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