Capping protein is dispensable for polarized actin network growth and actin-based motility

Majdouline Abou-Ghali1,2, Remy Kusters3, Sarah Körber4, John Manzi1,2, Jan Faix4, Cécile Sykes1,2, and Julie Plastino1,2,

From the 1Laboratoire Physico-Chimie Curie, Institut Curie, PSL Research University, CNRS, Paris, France, the 2Sorbonne Université, Paris, France, the 3Centre de Recherche Interdisciplinaire, Université de Paris INSERM U1284, Paris, France, and the 4Institute for Biophysical Chemistry, Hannover Medical School, Hannover, Germany

Edited by Enrique M. De La Cruz

Heterodimeric capping protein (CP) binds the rapidly growing barbed ends of actin filaments and prevents the addition (or loss) of subunits. Capping activity is generally considered to be essential for actin-based motility induced by Arp2/3 complex nucleation. By stopping barbed end growth, CP favors nucleation of daughter filaments at the functionalized surface where the Arp2/3 complex is activated, thus creating polarized network growth, which is necessary for movement. However, here using an in vitro assay where Arp2/3 complex-based actin polymerization is induced on bead surfaces in the absence of CP, we produce robust polarized actin growth and motility. This is achieved either by adding the actin polymerase Ena/VASP or by boosting Arp2/3 complex activity at the surface. Another actin polymerase, the formin FMNL2, cannot substitute for CP, showing that polymerase activity alone is not enough to override the need for CP. Interfering with the polymerase activity of Ena/VASP, its surface recruitment or its bundling activity all reduce Ena/VASP’s ability to maintain polarized network growth in the absence of CP. Taken together, our findings show that CP is dispensable for polarized actin growth and motility in situations where surface-directed polymerization is favored by whatever means over the growth of barbed ends in the network.

The discovery two decades ago of a mix of purified proteins capable of sustaining actin-based motility in the test tube was a breakthrough for understanding how actin dynamics drive movement (1). This system consisted of bacteria propelled forward via the localized polymerization of an actin tail or “comet.” Use of variants of this system led, and continue to lead, to major discoveries in the biochemistry of motility and the mechanisms of force production (2–6). The first minimal protein mix consisted of filamentous actin, an actin polymerization nucleator (the Arp2/3 complex) and its activator on the surface of the bacterium, a depolymerization factor (ADF/cofilin) and a capping protein. More recent versions of this system include profilin-actin to more closely mimic in vivo conditions of high actin monomer concentrations, and often do not include ADF/cofilin, as filament disassembly to replenish monomer levels is not required in the profilin-actin system provided reaction times are kept short (7). However, capping activity has been confirmed to be absolutely essential for polarized actin network growth and actin-based motility induced by Arp2/3 complex filament nucleation in many studies (1, 4, 8–10). The one notable exception is when high amounts of the Arp2/3 complex are present (4). In this case comets indeed form, but motility is inefficient.

These experiments beg the question as to why capping activity is necessary for motility in most cases. Heterodimeric capping protein (CP) tightly binds actin filament barbed ends, stopping their growth. The Arp2/3 complex creates new filaments as branches off the sides of mother filaments, remaining anchored at the pointed end of the daughter filaments, which grow with classical barbed end kinetics in all directions due to random branch orientations (4). The presence of CP in this context results in almost all polymerization occurring via nucleation of new filaments since growing barbed ends are rapidly capped. When nucleation is occurring at a surface due to functionalization to recruit and activate the Arp2/3 complex there, this results in growth predominantly at the surface, and this polarized growth is what produces actin-based motility (4, 11). In the absence of CP, filaments are nucleated and grow at the surface, but also grow everywhere else in the network. This gives unpolarized growth that is not able to produce movement. In the one case mentioned above where CP is not necessary for movement, it appears that excess of the Arp2/3 complex present in the reaction boosts nucleation at the surface, providing some actin growth polarity and modest motility (4). It is of note that reconstituted motility based on another actin polymerization nucleator, formin, does not require CP and is in fact inhibited by it (12). Formin creates new filaments without branching and remains attached to the growing barbed end, where it enhances barbed end elongation in the presence of profilin, and interferes with CP binding (12–15). When formin is immobilized on a surface, formin’s mechanism of action means that filament growth is coupled to the surface, actin polymerization is polarized, and propulsion occurs without the need for CP, as opposed to motility based on nucleation by the Arp2/3 complex.

Similar to formins Ena/VASP proteins enhance barbed end elongation and impede CP binding to barbed ends, although Ena/VASP proteins are not actin polymerization nucleators in...
physiological salt conditions (16–18). However, the N-terminal EVH1 domain of Ena/VASP proteins is known to bind the proline-rich domain of the Arp2/3 complex activators WASP, WAVE, and ActA (19–21), and Ena/VASP proteins are clearly linked to enhanced Arp2/3 complex-based lamellipodial protrusion and motility in cells (20, 22–24). Here we sought to understand how barbed end elongators, like Ena/VASP proteins, synergize with the Arp2/3 complex. Employing an in vitro system to address this question, we fortuitously observed that the presence of Ena/VASP made CP unnecessary for polarized actin growth and movement, even though actin polymerization nucleation was occurring via the Arp2/3 complex. In fact we found that augmented surface polymerization by any means, including excess Arp2/3 complex as previously reported (4), made CP superfluous. CP is of course present in vivo and its contribution to regulating the available pool of actin monomers and controlling Arp2/3 complex-based force production has been established in exquisite detail (Refs. 7 and 25 and references therein). Based on this there is a general conception that capping activity is necessary for motility driven by Arp2/3 complex nucleation. We show here that conditions can be found where this is not the case.

Results and discussion  
**VASP protein can replace CP in a bead motility assay**

Our in vitro system consisted of profilin–actin, CP, the Arp2/3 complex and 1-μm diameter beads, coated with the pVCA domain of the human WASP protein, an activator of the Arp2/3 complex. We also used the mouse Ena/VASP protein VASP, which increased barbed end elongation by about 35% as measured by TIRF microscopy: at 1.5 μm profilin–actin, elongation speeds were 1.3 ± 0.4 μm/min without VASP and 1.8 ± 0.4 μm/min in the presence of VASP (p = 0.002) (Fig. S1). This is in the same range as the 20% enhancement reported for human VASP under comparable salt conditions, using a similar TIRF method (18) although much smaller than the 7-fold enhancement observed for Dictostelium VASP (17).

As has been observed in numerous previous studies in the presence of CP, actin comets formed on the beads and pushed them forward (Fig. 1A and Fig. S2), whereas in the absence of CP, no comets were observable (see Ref. 26 and references therein). Unexpectedly adding VASP to the assay instead of CP produced robust actin comet formation and bead motility (Fig. 1A and Fig. S2). Beads moved at speeds of about 0.68 ± 0.04 μm/min with VASP in the absence CP, and 0.5 ± 0.1 μm/min with CP in the absence of VASP, comparable to what has been observed previously in the reconstituted extract system (20, 27). In the past, Ena/VASP proteins in the presence of CP have been studied extensively using bead or bead-type systems, and found to enhance Arp2/3 complex-based bead motility (20, 28–30). In these cases Ena/VASP was recruited to the surface, where it exercised its barbed end elongation enhancement activity on freshly-nucleated barbed ends since all other barbed ends were capped by CP. In the absence of CP, VASP would enhance barbed end elongation everywhere in the network, not just at the surface, so it was perplexing as to how VASP in the absence of CP could rescue polarized actin growth and movement.

**VASP restores polarized actin network growth in the absence of CP**

To shed light on how VASP could substitute for CP, we turned to a two-color approach where actin assembly was initiated in one color of actin (labeled with Alexa 594), allowed to polymerize, chased with another color (labeled with Alexa 488), and then observed. This is a classical way to identify where polymerizing ends are located in a network by following the incorporation of the second color (9, 10). For these experiments, we used large beads (4.5-μm diameter) to slow down comet formation as the time for comet formation increases with the size of the bead (10). This allowed us to examine actin cloud formation at the bead surface, as clouds never completely polarized to form comets on these beads although some asymmetric clouds were observed. In the presence of CP, the new (green) actin was enriched at the surface, distinct from the older (magenta) actin layer (Fig. 1B and Fig. S3). We called this separation of colors “color segregation,” and this indicated polarized actin growth at the bead surface, as occurs in actin-based motility. Adding VASP in the absence of CP gave a similar result (Fig. 1B and Fig. S3). In the absence of CP and VASP, overlap of the two colors was complete (Fig. 1B and Fig. S3), consistent with unpolarized actin growth where uncapped barbed ends in the body of the network were polymerizing, in addition to new branches forming at the surface. We quantified color segregation for a whole population of beads: without CP, only 13% of beads displayed color segregation (n = 90), whereas CP addition or VASP addition without CP produced segregated colors on 66% (n = 41) and 95% (n = 93) of beads, respectively.

**Increasing Arp2/3 complex activity also restores polarized actin network growth in the absence of CP**

We confirmed previous results by observing that a 3-fold increase in Arp2/3 complex concentration could produce color segregation in no CP conditions on 71% of the beads (n = 55, Fig. 2A and Fig. S4). Along the same lines when beads were coated with a tetrameric form of pVCA (S-pVCA) that activated the Arp2/3 complex 4-fold more efficiently than GST-pVCA (Fig. S5), color segregation was observed in the absence of CP, despite normal Arp2/3 complex concentration (50 nm) on 62% of the beads (n = 37, Fig. 2A and Fig. S4). Multimeric forms of WASP, such as S-pVCA, are believed to be more effective for Arp2/3 complex activation due to increased affinity for the Arp2/3 complex (31). All together these results suggested that VASP was somehow enhancing Arp2/3 complex activity since VASP addition gave a similar result to increasing Arp2/3 complex concentration or activation.

**VASP can compensate for suboptimal concentrations of the Arp2/3 complex in the absence of CP**

To better understand the interplay between VASP and the Arp2/3 complex, we examined network polarity for a range of concentrations of the Arp2/3 complex and VASP in the absence of CP (Fig. 2B). At low concentrations of the Arp2/3 complex and VASP, color segregation was observed in 45% of the beads (n = 25, Fig. 2B). But at higher VASP concentrations, close to optimal, segregation was observed in 95% of the beads (n = 35, Fig. 2B). This suggested there was a threshold concentration of VASP at which the Arp2/3 complex, despite suboptimal concentrations, could still be activated to a level at which color segregation could be observed. At even higher concentrations of VASP, up to 100% of the beads displayed color segregation (n = 25, Fig. 2B). This was a threshold concentration of VASP at which the Arp2/3 complex, despite suboptimal concentrations, could still be activated to a level at which color segregation could be observed. At even higher concentrations of VASP, up to 100% of the beads displayed color segregation (n = 25, Fig. 2B). This was a threshold concentration of VASP at which the Arp2/3 complex, despite suboptimal concentrations, could still be activated to a level at which color segregation could be observed.
complex and VASP, beads displayed weak fluorescence and no color segregation. As described above, at high concentrations of the Arp2/3 complex alone, actin networks around the beads were polarized and colors were segregated. On the other hand, at lower concentrations of the Arp2/3 complex, addition of VASP restored surface-directed polymerization in a dose-dependent manner (Fig. 2B). VASP compensation for suboptimal concentrations of the Arp2/3 complex suggested the possibility that VASP was somehow enhancing Arp2/3 complex activity. In this context, it is of note that there is no known direct interaction between VASP and the Arp2/3 complex (32).

Effect of VASP on branching in the network

Increased Arp2/3 complex activity should translate to more branches in the actin network. We evaluated this using fluorescently-labeled Arp2/3 complex and actin (Fig. 2C). We observed that adding VASP in the absence of CP increased the total amount of actin around the beads at about 15 min incubation by 2.5-fold as compared with no VASP/+CP conditions. On the other hand, the difference in the total amount of Arp2/3 complex in the network was not significantly different (Fig. 2C). This indicated that VASP was not increasing Arp2/3 complex activity and the incidence of branching in the actin network. In fact increased amounts of actin and constant amounts of the Arp2/3 complex indicated that the network was less branched in the presence of VASP, in keeping with our and others’ previous results linking VASP to a reduction in filament branching (23, 29, 30, 33).

Another form of VASP, but not the elongator FMLN2, can maintain network polarity in the absence of CP

Having ruled out increased Arp2/3 complex activity to explain our results with VASP, we next wondered whether the ability to substitute for CP was specific to mouse VASP or could be generalized to other barbed end elongators, including other Ena/VASP proteins. To test nonmouse Ena/VASP proteins with a range of barbed end elongation enhancement capacities, we turned to human/Dictyostelium VASP chimeras, containing a high affinity G-actin–binding site and equipped with different multimerization domains that modulated their activities: the dimer (VASP-2M) increased barbed end elongation by about 50%, the tetramer by 4-fold and the hexamer by 6-fold (34). When applied to beads in the two-color experiment, VASP-2M had a color-segregating effect comparable to mouse VASP (Fig. 1C).
Despite the nucleation activity of this VASP protein, which produced filaments in the bulk thus inhibiting growth on the bead surfaces. Indeed the more active tetrameric and hexameric forms of VASP could not be examined in the two-color experiments as they nucleated drastically, preventing polymerization on the beads.

To test whether elongation enhancement in general could substitute for CP, we next examined the formin FMNL2,
known to be a weak nucleator but a good elongator in profilin-actin (35). With a truncated form of FMNL2, FMNL2-8P, containing the profilin-actin recruitment site and the catalytic FH2 domain, we confirmed an enhancement of filament elongation, although FMNL2-8P was less active than VASP (Fig. S6). When this formin was applied to beads in the absence of CP, color segregation was never observed (Fig. 3A). This result indicated that barbed end elongation enhancement alone was not sufficient to overcome the requirement for CP in polarized actin network growth. We also tested the mDia1-FH1-FH2, but this formin nucleated polymerization extensively in the bulk, preventing surface polymerization, as observed with multimeric forms of VASP, mentioned above.

Restoring polarity with VASP depends partly on its surface recruitment and tetramerization

VASP’s polymerase activity is known to come from cooperation between its F-actin–binding domain (FAB) and its G-actin–binding domain (GAB), where FAB targets VASP to barbed ends and GAB contributes its bound monomer to filament growth (17, 18, 34, 36–38). In keeping with this, deleting VASP’s FAB domain reduced color segregation to such an extent as to not be significantly different from adding nothing at all (23%, n = 69, Table 1). Surprisingly mutating VASP’s GAB domain had no effect on color segregation (88%, n = 86, Table 1). However, deleting the proline-rich (PP) domain, which binds profilin-actin, significantly reduced color segregation (78%, n = 68, Table 1). In our profilin-actin system, it seemed that PP played the major role for VASP polymerase activity.

VASP is recruited to pVCA-coated beads, via the interaction between VASP’s EVH1 domain and the proline-rich domain of pVCA (20). To test the possibility that VASP was exercising its effect via surface recruitment, we deleted the EVH1 domain of VASP. This mutant displayed significantly less color segregation than the WT (73%, n = 82, Table 1). Although diminished, the fact that there was considerable polarity maintenance in the presence of ΔEVH1-VASP suggested that there were

Figure 3. How the actin network grows with other elongators and over time. A, separate channels and overlay images are shown (first color magenta, representing actin Alexa 594, second color green, representing actin Alexa 488). Line scans are measured as indicated by white lines, fluorescence intensity (arbitrary units) is plotted versus distance from the bead center, and separation of magenta and green curve maxima is taken as a segregation event, indicative of polarized growth. Top panels: actin growth in the presence of 100 nM chimeric human/Dicystostelium VASP dimer (VASP-2M). Color segregation occurs on 80% of the beads (n = 88). Bottom panels: actin growth in the presence of 50 nM FMNL2-8P, which gave 0% color segregation (n = 44). Epifluorescence microscopy was used. Scale bar, 5 μm. B, evolution over time of the total fluorescence of the actin network (medial plane, spinning disc images) in no CP/37 nM VASP conditions (open symbols) and in 25 nM CP conditions (closed symbols). Linear fits are shown. The Arp2/3 complex concentration was 50 nM (n ≥ 8) for beads in each condition. S.D. are large because of low signal to noise in spinning disc slices, but differences between no CP/+VASP and +CP conditions are significant except for actin fluorescence at 1 min.

Table 1

| VASP Form Added (50 nM) | Segregation | Number of beads analyzed | p Value | Value |
|-------------------------|-------------|--------------------------|---------|-------|
| WT VASP                 | 95%         | 93                       | –       |       |
| ΔFAB-VASP               | 23%         | 69                       | p < 0.0001<sup>a</sup> |       |
| No addition             | 13%         | 90                       | p < 0.0001<sup>a</sup> |       |
| ΔGAB-VASP               | 88%         | 86                       | p = 0.09<sup>b</sup> |       |
| ΔPP-VASP                | 78%         | 68                       | p = 0.0012<sup>c</sup> |       |
| ΔEVH1-VASP              | 73%         | 82                       | p = 0.0001<sup>a</sup> |       |
| ΔTET-VASP               | 78%         | 117                      | p = 0.0005<sup>a</sup> |       |

Arp2/3 complex concentration is 50 nM.
<sup>a</sup>As compared with WT VASP.
<sup>b</sup>As compared with ΔFAB-VASP.
<sup>c</sup>Not significant.

Restoring polarity with VASP depends partly on its surface recruitment and tetramerization

VASP’s polymerase activity is known to come from cooperation between its F-actin–binding domain (FAB) and its G-actin–binding domain (GAB), where FAB targets VASP to barbed ends and GAB contributes its bound monomer to filament growth (17, 18, 34, 36–38). In keeping with this, deleting VASP’s FAB domain reduced color segregation to such an extent as to not be significantly different from adding nothing at all (23%, n = 69, Table 1). Surprisingly mutating VASP’s GAB domain had no effect on color segregation (88%, n = 86, Table 1). However, deleting the proline-rich (PP) domain, which binds profilin-actin, significantly reduced color segregation (78%, n = 68, Table 1). In our profilin-actin system, it seemed that PP played the major role for VASP polymerase activity.

VASP is recruited to pVCA-coated beads, via the interaction between VASP’s EVH1 domain and the proline-rich domain of pVCA (20). To test the possibility that VASP was exercising its effect via surface recruitment, we deleted the EVH1 domain of VASP. This mutant displayed significantly less color segregation than the WT (73%, n = 82, Table 1). Although diminished, the fact that there was considerable polarity maintenance in the presence of ΔEVH1-VASP suggested that there were

Figure 2. Interplay of Arp2/3 complex activity and VASP for polarized actin growth in the absence of CP. The extent of polarized actin growth on 4.5-μm beads is assessed using the two-color approach. A, separate channels and overlay images are shown (first color magenta, representing actin Alexa 594, second color green, representing actin Alexa 488). Line scans are measured as indicated by white lines, fluorescence intensity (arbitrary units) is plotted versus distance from the bead center, and separation of magenta and green curve maxima is taken as a segregation event, indicative of polarized growth. Top panels: actin growth in the presence of excess (150 nM) Arp2/3 complex in the absence of CP and VASP. Bottom panels: beads are coated with a tetrameric form of pVCA (streptavidin-pVCA or S-pVCA), which is a more effective activator of the Arp2/3 complex than GST-pVCA. The polymerization mix contains no CP and no VASP, and 50 nM Arp2/3 complex. B, actin growth in the presence of varying amounts of the Arp2/3 complex and VASP in the absence of CP. Overlay images are shown, % color segregation and number of beads analyzed is indicated on an image of each condition; images were chosen to represent the majority case for each condition. The phase space where segregation occurs more than 50% of the time is depicted by the open symbols. C, images of actin and Arp2/3 complex in 25 nM CP and no CP/37 nM VASP conditions at about 10 min reaction time. The Arp2/3 complex concentration is 50 nM. A medial plane is shown. Quantification of total fluorescent intensity in the actin and Arp2/3 complex channels (n ≥ 7). n.s. indicates a nonsignificant difference. A and B, epifluorescence microscopy, C, spinning disc microscopy. All scale bars, 5 μm.
compensatory mechanisms at play. Another mechanism for surface bias could stem from a preference of VASP for freshly polymerized ATP-actin, but it is known that VASP binding to actin is independent of its nucleotide state (18, 28).

It is known that VASP is able to bundle filaments via its tetramerization domain (TET) (39, 40). Bundling could hold filaments in an orientation more favorable for assembly toward the surface, an effect that would not be relevant for filaments growing in the bulk network. To test this idea, we deleted the TET domain and observed that indeed color segregation was significantly reduced compared with WT (78%, n = 117, Table 1). Because oligomerization also contributes to barbed end elongation enhancement activity, we could not rule out that this reduction was due to an effect on polymerase activity, but in conditions such as ours, where VASP molecules were clustered on a surface, TET would no longer be needed for optimal polymerase activity (17). Unfortunately it was not possible to observe the fine structure of actin filaments by transmission EM on bead surfaces due to the thickness of the sample, so we could not confirm changes in surface organization of filaments in the presence of VASP. However, we know from previous studies that VASP has the effect of aligning filaments in the direction of movement in actin comets (29). That said, ablating the bundling activity of VASP had only a minor effect on color segregation, and thus could not be the whole explanation. Indeed previous studies gave no indication that bundling proteins such as fascin and α-actinin could stand in for CP in actin-based motility (41).

In summary for the VASP mutants, removal of the F-actin–binding site drastically reduced color segregation, whereas altering the G-actin–binding site had no effect at all. Deleting the other functional domains (profilin-actin-binding, tetramerization, and surface recruitment) significantly reduced color segregation, but nevertheless, these deletions were able to support considerable network polarity. Overall these results indicated that there were potentially multiple ways for VASP to favor surface polymerization, enabling the polymerization of uncapped barbed ends at the bead surface to outstrip uncapped barbed end polymerization in the network, thus giving polarized actin network growth on large beads and actin-based motility on small beads.

**Temporal evolution of network growth in the absence of CP**

We developed a model of how actin polymerizes at a surface over time to evaluate the effect of removing CP. In our bead system, the formation of new filaments occurred exclusively at the bead surface for two main reasons. First the Arp2/3 complex is an inefficient nucleator unless activated by pVCA, which was present only on the bead surface. Second we used profilin-actin, which curbed the spontaneous nucleation of filaments away from the bead surface (7). Since the Arp2/3 complex nucleates new filaments as branches off the sides of existing mother filaments, the source term for new polymerizing filaments is therefore branch formation.

The growth of the actin network around the bead can then be described with three kinetic rates: $v_b$, the branching rate, $v_p$, the barbed end elongation rate of filaments, and $v_c$, the capping rate that terminates filament elongation. All of this can be combined into a simple growth model, expressing the temporal evolution of the total amount of actin in the network around a single bead, expressed as a volume ($V$). $V$ is a function of the number of polymerizing ends, $N_{act}$, $v_p$ and $a^3$, the volume added to a growing filament with the addition of each monomer (Equation 1).

$$\frac{dV}{dt} = v_p a^3 N_{act} \quad \text{(Eq. 1)}$$

$N_{act}$ in turn varies over time inversely to the capping rate and proportionally to the source term, $v_p n_s$, which is the number of activators (pVCA molecules) on a bead surface, $n_s$, multiplied by the branching rate (Equation 2).

$$\frac{dN_{act}}{dt} = -v_c N_{act} + v_p n_s \quad \text{(Eq. 2)}$$

Integrating Equation 2 and assuming that at time 0, $N_{act} = 0$ gives an expression for $N_{act}$ (Equation 3).

$$N_{act} = \frac{v_p n_s}{v_c} (1 - e^{-v_c t}) \quad \text{(Eq. 3)}$$

Plugging this expression for $N_{act}$ into Equation 1 and solving for $V(t)$ gives Equation 4.

$$V(t)_{+CP} = \frac{v_p v_p n_s}{v_c^2} (e^{-v_c t} + (v_c t - 1))a^3 \quad \text{(Eq. 4)}$$

In the absence of capping ($v_c = 0$) in Equation 2, $N_{act}$ equals $v_p n_s$ and $V(t)_{noCP}$ can then be expressed by Equation 5.

$$V(t)_{noCP} = \frac{v_p v_p n_s}{2} t^2 a^3 \quad \text{(Eq. 5)}$$

Equations 4 and 5 describe the amount of actin that forms on the bead surface as a function of time in the presence and absence of CP, respectively. From comparing these equations, we can see that the amount of actin is very highly impacted by the presence of capping ($v_c^2$ term in the denominator of Equation 4), and that the amount of actin grows quadratically with time in the absence of CP ($t^2$ in Equation 5), whereas approximately linearly with time in the presence of capping since the term $e^{-v_c t}$ is small.

To get an idea of order of magnitudes, taking literature values for $v_c$ and $a^3$ and our estimated or measured values for $v_p$, $v_p$, $v_p + VASP$ and $n_s$ (see “Experimental procedures”), we calculated that at the 2-min reaction time, + VASP/no CP beads should already have 6-fold more actin around them than +CP beads. Most of this effect derives from the absence of CP not the increased elongation in the presence of VASP.

We compared this estimate to experimental data. We measured actin accumulation over time and observed that at 2 min, there was a 3-fold difference between + VASP/no CP and +CP conditions (Fig. 3B), and even at longer incubation times and also as observed in Fig. 2C, + VASP/no CP did not increase quadratically as predicted. The discrepancy between model and experiments concerning quantification of total actin around
beads over time in the absence of CP is probably due to several factors. First the model probably overestimates actin assembly rates as it does not take into account local monomer depletion effects, shown to be important even in conditions of high monomeric actin in the bulk (6). Second the experimental measurement probably underestimates the amount of actin around the beads. A growing actin layer on a bead has a far-reaching actin cloud that is 10 times bigger than the part that is visible by fluorescence microscopy, and reduced CP conditions makes this effect even more pronounced (42). Although the differences in actin accumulation that we observed in the presence of CP versus in no CP/+ VASP conditions were of the right order of magnitude as compared with the model, the main conclusion from this analysis was that VASP’s modest barbed end elongation enhancement activity had a relatively minor effect on actin growth, overshadowed by the presence/absence of CP.

The novel role of VASP that we reveal in this study is its ability to render CP unnecessary for motility and polarized actin network growth on beads. VASP appears to achieve this by selectively promoting surface assembly in the presence of Arp2/3 complex nucleation via a combination of barbed end elongation enhancement, surface recruitment and actin filament bundling. This bias is enough to drastically change outcome and allow polarized actin network growth and motility in the absence of CP. Likewise we show that other conditions that increase surface polymerization rescue polarity, indicating that CP is not an absolute requirement for Arp2/3 complex-based polarized actin growth and motility.

**Experimental procedures**

**DNA and proteins**

Rabbit muscle actin, pyrene-labeled rabbit muscle actin, and porcine Arp2/3 complex were purchased from Cytoskeleton as lyophilized powder and resuspended as per the manufacturer’s instructions. Fluorescently-labeled (Alexa 488 and Alexa 594) rabbit muscle actin was purchased from Invitrogen. All other proteins were purified or labeled in-house. The Arp2/3 complex was fluorescently labeled by incubation with a 10-fold molar excess of Alexa 488 C5-maleimide on ice for 3 h. 1 mM DTT was added to quench the labeling and the protein was dialyzed overnight in 20 mM Tris, pH 7.4, 25 mM KCl, 0.25 mM DTT, 100 μM ATP, 1 mM MgCl₂, 0.5 mM EDTA, centrifuged to remove precipitate and frozen. The DNA constructs for untagged human profilin and GST-pVCA-WASP-His (human WASP, residues 150-502, equipped with a GST and His8 tag, called GST-pVCA) were gifts of T. Pollard (Yale University) and L. Blanchon (CEA Grenoble), respectively. Profilin was purified as described by Carvalho et al. (43) and GST-pVCA (20). The streptavidin-tagged pVCA-WASP-His construct (S-pVCA) was purified as described by Carvalho et al. (43).

The DNA constructs for mouse α1β2 CP and WT and mutant forms of mouse VASP were gifts from D. Schafer (University of Virginia), and the proteins were purified (44) for CP and as in Ref. 16 for VASP and VASP mutants. VASP proteins were further purified via FPLC using a Superdex 200 10/300 GL column (GE Healthcare). VASP mutants were ΔEVH1-VASP, lacking residues 1–114; ΔGAB-VASP, carrying the double point mutation R232E, K233E in the G-actin–binding site; ΔPP-VASP, lacking residues 156–207; ΔFAB-VASP, lacking residues 255–273; and ΔTET-VASP, lacking residues 331–375. mVASP concentrations were calculated with the tetramer molecular weight. The chimera VASP-2M was purified as in Ref. 34 and its concentration is represented in monomers. mDia1-FH1-FH2 was purified as in Ref. 45.

The GST-FMNL2-8P construct was the kind gift of J. Pernier (I2BC, Paris-Saclay) (21). The construct was transformed into Rosetta 2(DE3) pLysS Escherichia coli (Novagen), and grown in 2 liters of 2YT medium with antibiotics. Expression was induced with 1 mM isopropyl 1-thio-β-d-galactopyranoside, 20 °C, overnight. Cells were lysed by sonication in lysis buffer: 20 mM Tris, pH 7.5, 500 mM NaCl, 1 mM EDTA, and 1 mM DTT, supplemented with 1 mM phenylmethylsulfonyl fluoride and complete EDTA-free protease inhibitor mixture (Roche Applied Science). Protein was bound to Glutathione Sepharose 4 fast flow beads (GE Healthcare). Unbound proteins were washed away with lysis buffer, and bound proteins eluted in 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM DTT, 25 mM reduced glutathione, pH 7.5. For GST tag removal, a final concentration of 1 mM EDTA was added to samples, plus 50 μg of PreScission Protease. GST was cleaved overnight at 4 °C. The sample was further purified in a buffer of 20 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT, using the HiLoad Superdex 200 16/600 pg column. The peak containing FMNL2 was collected, frozen in liquid nitrogen, and stored at −80 °C. All protein concentrations were measured by Bradford assay.

**Bead preparation**

For bead assays carboxylate beads (Polysciences) were used. 9 μl of 2.5% bead suspension, 4.5-μm diameter, or 2 μl of 2.5% bead suspension, 1.0-μm diameter (total surface area 3 cm²) were coated in 40 μl of 2 μM GST-pVCA-WASP or S-pVCA-WASP in Xb (10 mM HEPES, pH 7.5, 0.1 mM KCl, 1 mM MgCl₂, and 0.1 mM CaCl₂). The reaction was mixed in a thermomixer for 20 min at 18 °C and 1000 rpm. After coating, the bead surface was blocked by washing twice with 1% BSA, Xb buffer. The coated beads were resuspended in 120 μl of Xb, 1% BSA and stored on ice for experiments.

**Actin polymerization on beads**

Actin was thawed, diluted to ~20-30 μM in G-buffer (2 mM Tris, 0.2 mM CaCl₂, 0.2 mM DTT, 0.2 mM ATP, pH 8.0), and allowed to depolymerize at 4 °C for at least 2 days and then kept on ice and used for several weeks. Profilin, CP, the Arp2/3 complex, and KCl were all diluted in MB13 buffer (10 mM HEPES, 1.5 mM ATP, 3 mM DTT, 1.5 mM MgCl₂, 1 mM EGTA, 50 mM KCl, 1% BSA, pH 7.5). VASP proteins were diluted in VASP buffer (20 mM imidazole, 200 mM KCl, 1 mM EGTA, 2 mM MgCl₂, 1 mM DTT, pH 7.0).

The *in vitro* actin polymerization reaction mix contained: 0.2 μl of coated beads (~0.005 cm² of surface), 50 nM Arp2/3 complex, 5 or 15 μM profilin (either a 1:1 ratio or a 1:3 ratio), and 5 μM G-actin, with or without 25 nM CP and/or 50 nM VASP, except for the phase diagram experiments where the concentrations of the Arp2/3 complex and VASP were varied, and the
spinning disc experiments that were performed at 37 nM VASP. The final KCl concentration was adjusted to 86 mM by addition of KCl in MB13. The final reaction volume was 8.4 µl. The entire reaction was spotted on a glass slide, covered with a coverslip (18 × 18 mm), and sealed with vaseline/lanolin/paraffin (1:1:1). For timed experiments, the stopwatch was started upon addition of actin, which was always added last.

**Two-color experiments**

Reaction conditions were as described above, but with Alexa 488- or Alexa 594-labeled actin added to the diluted unlabeled actin solution in G-buffer to a final concentration of 10% labeled actin, and allowed to depolymerize before use. For the two-color experiment, a half-batch (4.1 µl) of actin polymerization reaction mix + beads (see above) was prepared with Alexa 594-labeled actin and allowed to polymerize in the tube at room temperature until actin polymerization was well underway (anywhere from 5 to 20 min depending on conditions). This reaction was then mixed with a second reaction mix (8.4 µl) containing Alexa 488-labeled actin, but no beads. The entire mixture was spotted on a slide and photographed over time, with the best color segregation observed at 10-20 min reaction time (images shown in the main figures), although clouds continued to grow for several hours.

**Bead observation and data processing**

Phase-contrast and epifluorescence microscopy images were obtained on an Olympus IX70 inverted microscope with a ×100 oil-immersion objective and CoolSnap CCD camera (Photometrics). Spinning disc images were obtained on an inverted confocal spinning disc microscope from Nikon using a ×100 oil objective and a CoolSNAP HQ2 camera (Photometrics). Phase-contrast and fluorescence quantification was done using MetaMorph software (Universal Imaging). For speed estimations, pictures of 1-µm beads with comets were taken randomly over the whole slide for about 20 min, lengths were plotted versus time, and the slopes were taken as the average speed. For two-color experiments, pictures of beads were taken randomly over the whole slide. For each bead, 2 pictures were taken, one for green fluorescence and one for red fluorescence, and the two pictures were overlaid in MetaMorph. The line scan function of MetaMorph was used on the combined images, drawing a line from the center of the bead toward the outside. This gave the intensity of each pixel in the red and green channel with respect to its position along the line, and was plotted after subtracting the background, taken at the furthest extreme of the line scan from the bead surface. Line scans were drawn by hand at a location that gave the best color segregation profile regardless of conditions.

For Arp2/3 complex quantification coupled with actin measurements, bead stacks were imaged by spinning disc at ~10-20 min reaction time, where growth had plateaued. A single plane where the bead appeared largest was taken, and densities were evaluated in MetaMorph by drawing a doughnut shape that surrounded the bead and included 1 µm of the network around the bead. Background was subtracted. For temporal evaluation of actin growth, bead stacks were taken over time by spinning disc, and the maximum intensity projection of the two central planes (where the bead appeared the largest) was analyzed using a Matlab script to measure the total fluorescence intensity in the entire image (one bead per field). For all data significant differences were calculated and p values reported using the Student’s t test for comparison of averages, and a χ-squared test for comparison of %. p < 0.05 was taken as significant.

**Actin polymerization assessment by pyrene assay**

For assessment of GST-pVCA and S-pVCA activity, the pyrene assay mix (60 µl final volume) contained 50 nM Arp2/3 complex, 15 µM profilin, 5 µM actin (~5% labeled with pyrene, diluted to 30 µM in G-buffer and allowed to depolymerize for at least 2 days before use), and 86 mM KCl in MB13 buffer. GST-pVCA and S-pVCA were diluted in MB13. For assessment of FMNL2-8P activity, the same mix was used, minus the Arp2/3 complex. Seeds were formed by allowing actin to polymerize to a plateau in the absence of profilin, and then kept on ice. Approximately 0.4 µM actin filaments was used to seed each reaction. For all curves, as soon as monomeric actin was added, the mix was placed in a glass cuvette and the fluorescence intensity (excitation 365 nm, emission 407 nm, excitation slit 5 nm, emission slit 5 nm) was measured every second using a fluorimeter (Cary) thermostatted at 20°C. Kaleidagraph was used to plot the data. The concentration of barbed ends was calculated with the equation: [b.e.] = (elongation rate µM s⁻¹)/(k⁺[actin monomers]), where elongation rate at half-maximum was converted from absorbance units to micromolar based on the curve plateau assuming all actin was in filamentous form at this point, using 2.5 µM as the actin monomer concentration at half-maximum and taking k⁺ as ~10 µM⁻¹ s⁻¹ (46, 47).

**Single filament assay by TIRF microscopy**

Glass coverslips were cleaned in a glass holder using 1 M NaOH and sonication for 15 min, then washed in water, sonicated again in 96% ethanol for 15 min, washed in water, and dried using pressure nitrogen flow. Clean coverslips were assembled into chambers where the sample was sandwiched between an 18 × 18- and a 24 × 50-mm coverslip separated by double-sided tape. Experiments were performed using an Eclipse Ti Inverted Microscope with a ×100 oil immersion objective and a Quantum 512SC camera (Photometrics). Actin polymerization mix contained 1.5 µM Alexa 488-labeled actin (15% labeling), 1.5 µM profilin, 86 mM KCl, 0.2% 1,4-diazabicyclo[2,2,2]octane, and 4% methylcellulose in MB13. VASP was added at 37 nM. Samples were flowed into the chambers and sealed with vaseline/lanolin/paraffin. Image acquisition started 1 min after the start of polymerization in the chamber. Images were collected at 1-s intervals for 15 min. Actin filament lengths were measured over time, and converted to rate constants by considering that 1 µM represented 370 subunits of actin (48). At least 16 filaments were measured for each condition.

**Constants used in the calculations**

The rate of capping, νc, was taken as 0.065 s⁻¹, based on our CP concentration of 25 nM and the rate constant of 2.6 × 10⁶

---

**Capping Activity Can Be Rendered Superfluous**

Glass coverslips were cleaned in a glass holder using 1 M NaOH and sonication for 15 min, then washed in water, sonicated again in 96% ethanol for 15 min, washed in water, and dried using pressure nitrogen flow. Clean coverslips were assembled into chambers where the sample was sandwiched between an 18 × 18- and a 24 × 50-mm coverslip separated by double-sided tape. Experiments were performed using an Eclipse Ti Inverted Microscope with a ×100 oil immersion objective and a Quantum 512SC camera (Photometrics). Actin polymerization mix contained 1.5 µM Alexa 488-labeled actin (15% labeling), 1.5 µM profilin, 86 mM KCl, 0.2% 1,4-diazabicyclo[2,2,2]octane, and 4% methylcellulose in MB13. VASP was added at 37 nM. Samples were flowed into the chambers and sealed with vaseline/lanolin/paraffin. Image acquisition started 1 min after the start of polymerization in the chamber. Images were collected at 1-s intervals for 15 min. Actin filament lengths were measured over time, and converted to rate constants by considering that 1 µM represented 370 subunits of actin (48). At least 16 filaments were measured for each condition.

**Constants used in the calculations**

The rate of capping, νc, was taken as 0.065 s⁻¹, based on our CP concentration of 25 nM and the rate constant of 2.6 × 10⁶
Capping Activity Can Be Rendered Superfluous

M⁻¹ s⁻¹ (49). The volume added per monomer addition event, \(a^3\), was (2.7 nm)³ (48). The number of activators on a bead surface, \(n_a\), was calculated as 2.5 × 10⁶ from the pVCA spacing of 5 nm (50) and the surface area of a 4.5-μm diameter bead. The polymerization rate, \(v_p\), was calculated as 28 s⁻¹ in the absence of VASP and 37 s⁻¹ in the presence of VASP, \(v_p^{VASP}\). These values derive from the monomeric actin concentration in our assay of 5 μM and our measured barbed end \(k_0\) of around 5.5 μM⁻¹ s⁻¹ in the absence of VASP and 7.4 μM⁻¹ s⁻¹ in the presence of VASP, measured by TIRF microscopy. 5.5 μM⁻¹ s⁻¹ is half the value observed without profilin (about 10 μM⁻¹ s⁻¹ (47)), due to the inhibitory effect of excess or stoichiometric profilin on barbed end polymerization (18, 51). The branching monomer size, and branching: \(\xi = av_p/v_b\), giving \(v_b\) as 1.5 s⁻¹. This value of \(v_b\) was used for +VASP conditions as well, as there are indications that mesh size is larger in the presence of VASP (23, 29) thus potentially compensating for increased \(v_p\).

Data availability

All data associated with this paper are included in the manuscript and in the supplementary information.

Acknowledgments—We warmly acknowledge Olivier Renaud et Olivier Leroy, of the Cell and Tissue Imaging Platform (member of France BioImaging, ANR-10-INBS-04) of the Genetics and Developmental Biology Department (UMR3215/U934) of Institut Curie for help with the light microscopy.

Author contributions—M. A.-G., C. S., and J. P. conceptualization; M. A.-G., R. K., S. K., and J. P. formal analysis; M. A.-G. and J. P. validation; M. A.-G. R. K., S. K., J. M., and J. P. investigation; M. A.-G., R. K., and J. P. visualization; M. A.-G., R. K., S. K., J. M., and J. P. methodology; R. K. software; J. M. and J. F. resources; J. F., C. S., M. A.-G., R. K., S. K., J. M., and J. P. investigation; M. A.-G., R. K., S. K., and J. P. formal analysis; M. A.-G. and J. P. validation; M. A.-G. and J. P. writing-review and editing; C. S. and J. P. funding acquisition; J. P. supervision; J. P. writing-original draft; J. P. project administration.

Funding and additional information—This work was supported by grants from the program “Investissements d’Avenir” from the French Government and implemented by ANR Grants ANR-10-LABX-0038 and ANR-10-IDEX-0001 PSL and a post-Ph.D. grant (to M. A.-G.). J. P. was supported by Fondation pour la Recherche Médicale Grant DEQ20120323737 and Fondation ARC Grants PA 20151203487 and PA 20191209604. M. A.-G. was supported by a Ph.D. fellowship from La Ligue Contre le Cancer. S.K. was supported by the ERASMUS+ Student Mobility Placement program. R. K. was supported by the Bettencourt Schueller Foundation long-term partnership and partly by the CRI Research Fellowship program.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: CP, capping protein; TIRF, total internal reflection fluorescence; FAB, F-actin–binding protein; GAB, G-actin–binding domain; PP, polyproline; TET, tetramerization; GST, glutathione S-transferase.

References

1. Loisel, T. P., Boujemaa, R., Pantaloni, D., and Carlier, M. F. (1999) Reconstitution of actin-based motility of Listeria and Shigella using pure proteins. Nature 401, 613–616 CrossRef Medline
2. Cameron, L. A., Footer, M. J., Van Oudenaarden, A., and Theriot, J. A. (1999) Motility of Acta protein-coated microspheres driven by actin polymerization. Proc. Natl. Acad. Sci. U.S.A. 96, 4908–4913 CrossRef
3. Bernheim-Grosawasser, A., Wiesner, S., Golsteyn, R. M., Carlier, M.-F., and Sykes, C. (2002) The dynamics of actin-based motility depend on surface parameters. Nature 417, 308–311 CrossRef Medline
4. Achard, V., MartiJ, J.-L., Michelot, A., Guérin, C., Reymann, A.-C., Blanchon, L., and Boujemaa-Paterski, R. (2010) A “primer”-based mechanism underlies branched actin filament network formation and motility. Curr. Biol. 20, 423–428 CrossRef Medline
5. Reymann, A.-C., Boujemaa-Paterski, R., Martiel, J.-L., Guérin, C., Cao, W., Chin, H. F., De La Cruz, E. M., Théry, M., and Blanchon, L. (2012) Actin network architecture can determine myosin motor activity. Science 336, 1310–1314 CrossRef Medline
6. Boujemaa-Paterski, R., Suarez, C., Klar, T., Zhu, J., Guérin, C., Mogilner, A., Théry, M., and Blanchon, L. (2017) Network heterogeneity regulates steering in actin-based motility. Nat. Commun. 8, 655 CrossRef Medline
7. Plastino, J., and Blanchon, L. (2019) Dynamic stability of the actin ecosys- tem. J. Cell Sci. 132, jcs219832 CrossRef
8. Dürre, K., Keber, F. C., Bleicher, P., Brauns, F., Cyron, C. J., Faix, J., and Bausch, A. R. (2018) Capping protein-controlled actin polymerization shapes lipid membrane. Nat. Commun. 9, 1630 CrossRef Medline
9. Akin, O., and Mullins, R. D. (2008) Capping protein increases the rate of actin-based motility by promoting filament nucleation by the Arp2/3 complex. Cell 133, 841–851 CrossRef Medline
10. van der Gucht, J., Paluch, E., Plastino, J., and Sykes, C. (2005) Stress release drives symmetry breaking for actin-based movement. Proc. Natl. Acad. Sci. U.S.A. 102, 7847–7852 CrossRef Medline
11. Sykes, C., and Plastino, J. (2010) Actin filaments up against a wall. Nature 464, 365–366 CrossRef Medline
12. Romero, S., Le Claire, C., Didry, D., Egle, C., Pantaloni, D., and Carlier, M.-F. (2004) Formin is a processive motor that requires profilin to accelerate actin assembly and associated ATP hydrolysis. Cell 119, 419–429 CrossRef Medline
13. Kovar, D. R., Harris, E. S., Mahaffy, R., Higgs, H. N., and Pollard, T. D. (2006) Control of the assembly of ATP- and ADP-actin by formins and profilin. Cell 124, 435–445 CrossRef Medline
14. Kovar, D. R., Kuhn, J. R., Tichy, A. L., and Pollard, T. D. (2003) The fission yeast cytokinesis formin Cdc12p is a barbed end actin filament capping protein gated by profilin. J. Cell Biol. 161, 875–887 CrossRef Medline
15. Kovar, D. R., and Pollard, T. D. (2004) Insertional assembly of actin filament barbed ends in association with formins produces piconewton forces. Proc. Natl. Acad. Sci. U.S.A. 101, 14725–14730 CrossRef Medline
16. Barzik, M., Kotova, T. L., Higgs, H. N., Hazelwood, L., Hanein, D., Gertler, F. B., and Schafer, D. A. (2005) Ena/VASP proteins enhance actin polymerization in the presence of barbed end capping proteins. J. Biol. Chem. 280, 28653–28662 CrossRef Medline
17. Breitsprecher, D., Kiselevetter, A. K., Linker, J., Urbanke, C., Resch, G. P., Small, J. V., and Faix, J. (2008) Clustering of VASP actively drives processes, WH2 domain-mediated actin filament elongation. EMBO J. 27, 2943–2954 CrossRef Medline
18. Hansen, S. D., and Mullins, R. D. (2010) VASP is a processive actin polymerase that requires monomeric actin for barbed end association. J. Cell Biol. 191, 571–584 CrossRef Medline
19. Castellano, F., Le克莱恩, C., Patin, D., Carlier, M.-F., and Chavrier, P. (2001) A VASP-VASP complex regulates actin polymerization at the plasma membrane. EMBO J. 20, 5603–5614 CrossRef Medline
20. Havrylenko, S., Noguera, P., Abou-Ghali, M., Manji, Z., Faqir, F., Lamora, A., Guérin, C., Blanchon, L., and Plastino, J. (2015) WAVE binds Ena/
